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The biogeochemistry and bioremediation of uranium and other priority radionuclides

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ABSTRACT

Microbial metabolism has the potential to alter the solubility of a broad range of priority radionuclides, including uranium, other actinides and fission products. Of notable interest has been the biostimulation of anaerobic microbial communities to remove redox-sensitive radionuclides such as uranium U(VI) from contaminated groundwaters at nuclear sites. Particularly promising are bioreduction processes, whereby bacteria enzymatically reduce aqueous U(VI) to insoluble U(IV) coupled to oxidation of an organic electron donor; and uranium phosphate biomineralisation, in which bacterial phosphatase activity cleaves organophosphates, liberating inorganic phosphate that precipitates with aqueous U(VI) as uranyl phosphate minerals. Here we review the mechanisms of uranium bioreduction and phosphate biomineralisation and their suitability to facilitate long-term precipitation of uranium from groundwater, with particular focus on in situ trials at the US Department of Energy field sites. Redox interactions of other priority radionuclides (technetium, neptunium, plutonium, americium, iodine, strontium and caesium) are also reviewed.

Contents

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1. Introduction

Decades of nuclear activities have left a legacy of environmental contamination. Elevated concentrations of uranium and other radionuclides are present in mining and milling areas, at sites where uranium ore was processed, and where uranium was enriched. This contamination potentially represents an uncontrolled source of radiation, and therefore regulatory bodies may require it to be remediated to acceptable levels.

The mobility of uranium in the environment depends on its speciation and redox state (Fig. 1). It is present as mobile U(VI) in oxidising conditions, predominantly as the uranyl ion (UO_2^{2+}) or hydroxyl complexes below ~pH 6.5, or as uranyl carbonate complexes at higher pH

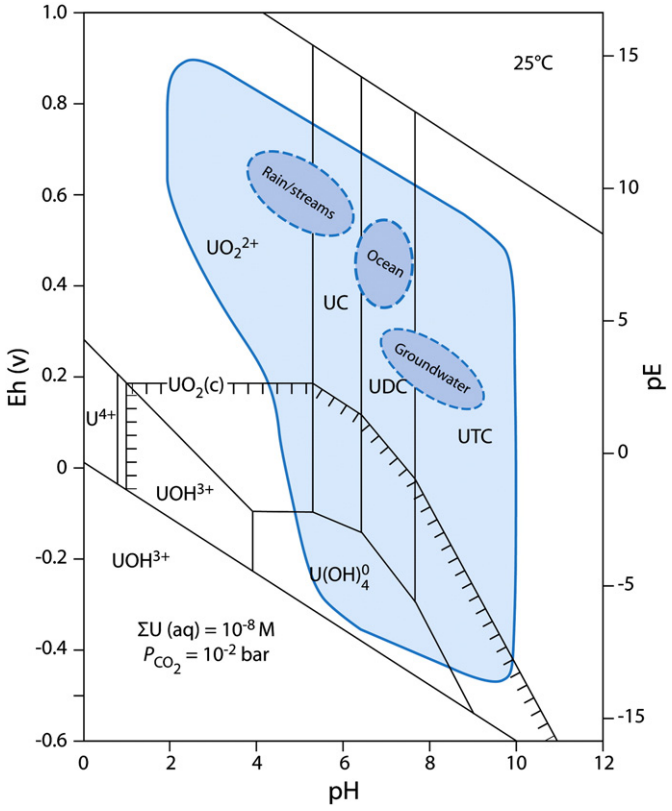

Fig. 1. Eh–pH diagram for aqueous species in the U–O_2–CO_2–H_2O system in pure water at 25 °C and 1 bar total pressure for $\Sigma U = 10^{-8}$ M and a typical groundwater CO_2 pressure of $PCO_2 = 10^{-2.0}$ bar, after Langmuir (1997). UC, UDC and UTC represent the aqueous complexes $UO_2CO_3^0$, $UO_2(CO_3)_2^{2-}$ and $UO_2(CO_3)_3^{4-}$. The position of the $UO_{2(c)}$ solid solution boundary for $\Sigma U = 10^{-8}$ M is stippled. The blue area represents the range of conditions of common natural waters, after Ewing (2010).

(Choppin et al., 2002). In the absence of carbonate, the uranyl ion and its complexes sorb strongly onto the surface of iron oxides and organics (Hsi and Langmuir, 1985; Andersson et al., 2001; Koch-Steindl and Pröhl, 2001) and onto the edge sites of clay minerals (Pabalan et al., 1998; Davis et al., 2004). Sorption decreases in the presence of complexing ligands such as humic and fulvic acids, and in the presence of competing cations such as Ca^{2+} and Mg^{2+} (Siegal and Bryan, 2003). Under reducing conditions, relatively insoluble and immobile U(IV) predominates, typically as the mineral uraninite, but recently a different, non-uraninite U(IV) phase has been reported (Bernier-Latmani et al., 2010; Bargar et al., 2013). U(V) is generally considered to be transient although evidence is emerging to suggest that it might be stable for periods of weeks under certain conditions (Docrat et al., 1999; Behrends et al., 2012; Ilton et al., 2012). Depleted uranium metal, used in anti-tank penetrators and present in the environment as a legacy of military activities, is relatively immobile, but may be oxidised to uraninite and consequently U(VI) depending on the redox conditions (UNEP, 2003; Parrish et al., 2008; Handley-Sidhu et al., 2010).

Biogeochemical interactions play a key role in controlling the speciation and mobility of uranium and other redox sensitive radionuclides (such as Tc, Np and Pu), through direct metabolic processes such as microbial respiration, or indirectly by changing ambient redox/pH conditions, producing ligands or new biominerals, or altering mineral surfaces. In addition to controlling radionuclide mobility via "natural attenuation", these biogeochemical processes can be stimulated to accelerate clean-up of contaminated environments through bioremediation. This review focuses on microbe–radionuclide interactions, how they may control radionuclide (especially uranium) mobility in natural environments, and how they can be applied to bioremediate legacy metal and radionuclide contamination.

2. Microbe–radionuclide interactions

Microbial interactions with uranium and other radionuclides have been documented extensively e.g. Gorby and Lovley (1992), Lloyd and Gadd (2011), Lloyd and Macaskie (2000), Lloyd and Renshaw (2005), Lovley et al. (1991) and Merroun and Selenska-Pobell (2008). Most study the interactions between uranium and bacteria; these are the focus of this review. There is emerging interest in the use of microbial "bioleaching" mechanisms to extract uranium from low grade ores e.g. Choi et al. (2005) and Qiu et al. (2011), although these are not covered further in this review. Preventing uncontrolled dispersion and transport of radionuclides in groundwater is the overarching remediation goal at many nuclear sites. Stimulating bacterial interactions to fix aqueous uranium into insoluble minerals in situ may provide a relatively inexpensive and non-intrusive solution to remediating radionuclide contamination. The mechanisms of the different microbe–uranium interactions are illustrated in Fig. 2 and discussed below, along with their suitability to facilitate long-term uranium removal.

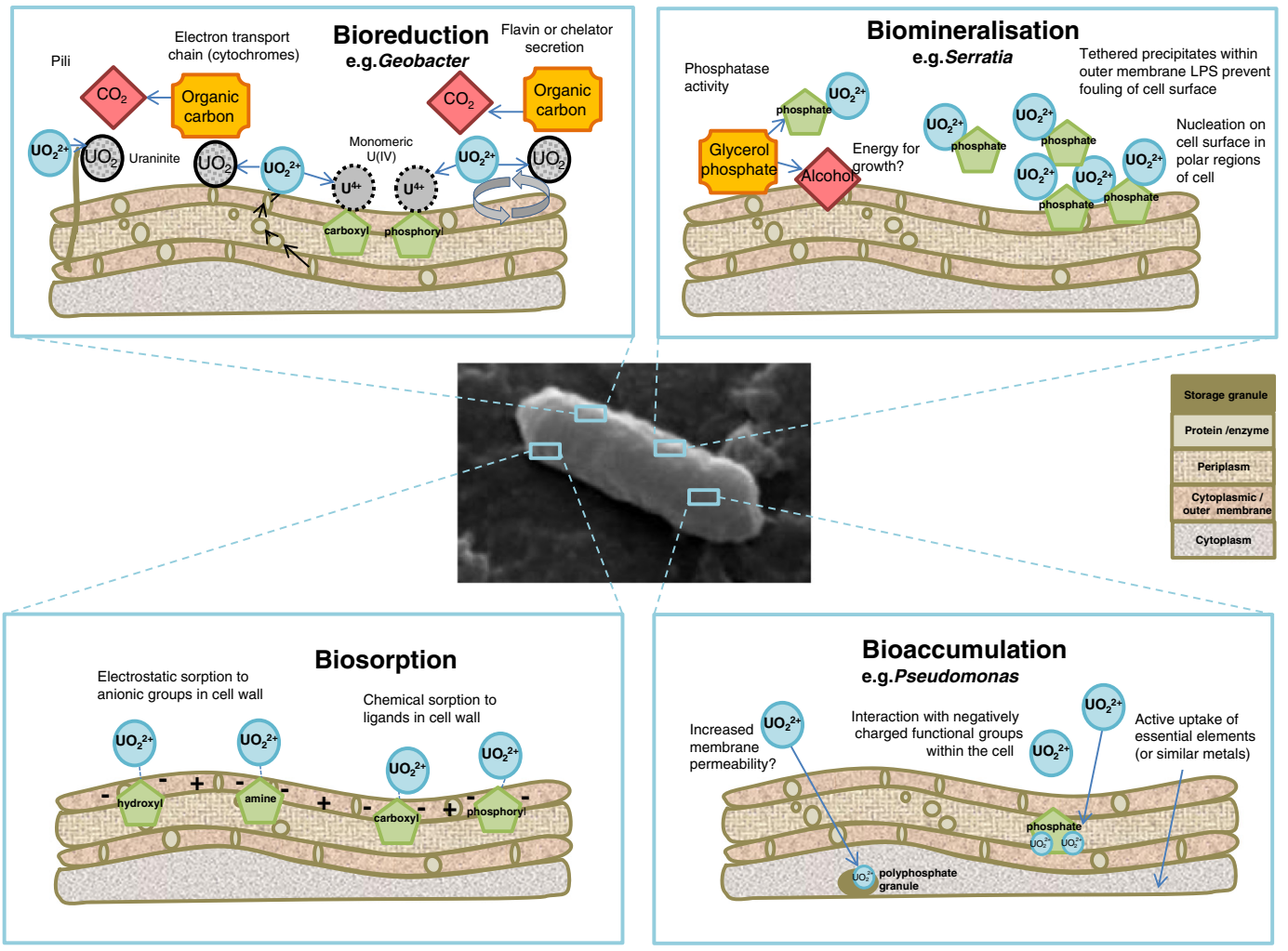

Fig. 2. Schematic illustrating the mechanisms of microbe–uranium interactions. Examples of recent or key references for these mechanisms include: bioreduction (Lovley et al., 1991; Bernier-Latmani et al., 2010; Brutinel and Gralnick, 2012; Williams et al., 2012), biomineralisation (Macaskie et al., 1992, 2000; Beazley et al., 2011), biosorption (Beveridge and Murray, 1980; Gadd, 2009) and bioaccumulation (Choudhary and Sar, 2011).

2.1. Bioreduction

In the absence of oxygen, bacteria are able to respire different electron acceptors to gain energy for metabolism. As anoxia progresses, the most energetically favourable electron acceptors are used in sequence, starting with the reduction of nitrate, then proceeding through Mn(IV), Fe(III) and sulfate, and finally the reduction of carbon dioxide to produce methane. While this sequence is generally correct for the natural environment, it should be noted that under certain situations, such as when organic matter is in abundance, nitrate- and metal-reduction, or metal- and sulfate-reduction could potentially occur concurrently (Madden et al., 2007; Williams et al., 2011). At circumneutral pH, U(VI) has a similar redox couple to Fe(III), and Fe(III)-reducing bacteria are able to respire U(VI) as an alternative electron acceptor, reducing it to insoluble U(IV) (Lovley et al., 1991). Other groups capable of U(VI) reduction include sulfate-reducing bacteria (Lovley and Phillips, 1992a), fermentative bacteria (Francis et al., 1994), acid-tolerant bacteria (Shelobolina et al., 2004) and myxobacteria (Wu et al., 2006); some conserving energy for growth, others with no energy gain (Merroun and Selenska-Pobell, 2008).

Uranium bioreduction has been proposed as a bioremediation technique, stimulated by adding an electron donor to promote enzymatic reduction of aqueous U(VI) to insoluble U(IV). It has been demonstrated in laboratory experiments representative of UK conditions (Wilkins et al., 2007; Begg et al., 2011; Law et al., 2011) and also in situ in the USA (Istok et al., 2004; Wu et al., 2007; Williams et al., 2011). The speciation of bioreduced uranium is often stated to be uraninite [UO_2] (Lovley and Phillips, 1992a; Suzuki et al., 2002) however; more recently other U(IV) forms have been identified as end-points (Kelly et al., 2008; Bernier-Latmani et al., 2010; Alessi et al., 2012). Most work has focussed on removal of aqueous U(VI) from solution, however, U(VI) may be present in the solid phase or sorbed to minerals. Microbial reduction of poorly soluble U(VI) as uramphite [$(NH_4)(UO_2)(PO_4).3H_2O$], was demonstrated using *Thermoterrabacterium ferrireducens* (Khijniak et al., 2005) (since reclassified as *Carboxydothermus ferrireducens* (Slobodkin et al., 2006)), and as metaschoepite [$UO_3 \cdot 2H_2O$] using *Shewanella putrefaciens* CN32 (Fredrickson et al., 2000), while bioreduction of sorbed U(VI) has been shown in natural soils (Begg et al., 2011; Law et al., 2011) and with synthetic and natural iron minerals (Jeon et al., 2004). Abiotic reduction of U(VI) is possible by Fe(II) minerals (Regenspurg et al., 2009; Hyun et al., 2012; Latta et al., 2012; Singer et al., 2012; Fox et al., 2013) and biominerals (O'Loughlin et al., 2010; Veeramani et al., 2011, 2013), however, the majority of studies have suggested that direct enzymatic reduction is the dominant mechanism mediating U(VI) reduction under ambient environmental conditions (Williams et al., 2012; Bargar et al., 2013). Potential concerns associated with the use of bioreduction as a remediation technique stem from whether reduced U(IV) will be stable over long time periods, particularly if the environmental conditions change, for example to oxidising conditions (Senko et al., 2002).

2.2. Biomineralisation

Biomineralisation refers to the process by which metals precipitate with microbially generated ligands such as sulfide or phosphate, or as carbonates or hydroxides in response to localised alkaline conditions at the cell surface. Uranium biomineralisation has been proven using a *Citrobacter* species (Macaskie et al., 1992), since reclassified as a *Serratia* species (Pattanapipitpaisal et al., 2002). When supplied with glycerol phosphate, the cell phosphatase activity cleaved the organic phosphate to release inorganic phosphate, which precipitated with U(VI) as extracellular hydrogen uranyl phosphate minerals [HUO_2PO_4]. This has also been demonstrated using an environmental isolate from the US DOE Oak Ridge site (Beazley et al., 2007), and by a *Pseudomonas* species when supplied with a tributylphosphate donor (Thomas and Macaskie, 1996). Microbial cells that were entirely covered with uranium phosphate minerals have been observed in uraniferous soils, suggesting bacterial biomineralisation was occurring naturally in this system (Mondani et al., 2011).

A simpler approach would be to add inorganic phosphate directly to uranium contaminated groundwater, however, as phosphate is very reactive it is likely to precipitate rapidly with aqueous metals leading to clogging and limiting dispersion into the environment (Wellman et al., 2006). Stimulating bacterial phosphatase activity to liberate phosphate under controlled conditions limits the ingrowth of phosphate to the system to the rate of bacterial hydrolysis of organophosphate, thus avoiding clogging of the injection location with metal phosphate minerals. Furthermore, biomineralisation is often more efficient than chemical precipitation in dilute solutions because the ligands are concentrated near the cell surface, which provide nucleation foci for precipitation (Lloyd and Macaskie, 2000).

A potential problem with biomineralisation is that rapid precipitation of metals around the cell surface could in principle create a barrier to cell metabolism, although this has not been directly observed (Lloyd and Macaskie, 2000). A recent review highlighted the contradiction between some studies which suggest that biomineralisation is a toxicity resistance mechanism, and others in which it is assumed to be detrimental to the cells (Benzerara et al., 2011). From the sparse evidence available, it appears that encrustation does not necessarily limit metabolic activity. In the *Serratia* system, images of the precipitates appear to show uranyl phosphates were deposited on the cell wall on one side of the cell, or 'tethered' within the lipopolysaccharide preventing fouling of the cell surface (Macaskie et al., 2000). Bacteria may cause dissolution of uranyl phosphates such as autunite in phosphate limited systems (Smeaton et al., 2008). Other challenges may come from the cost of the organic phosphate donor, limiting the economic viability of biomineralisation as a bioremediation technique (Roig et al., 1995; Lloyd and Macaskie, 2000). Biominerals can act as nucleation foci for metal deposition; a process referred to as "microbially enhanced chemisorption of heavy metals" or MECHEM (Lloyd and Macaskie, 2000). For example, nickel can be removed from solution via intercalation into hydrogen uranyl phosphate (Bonthrone et al., 1996).

2.3. Bioaccumulation

Microbial cells are also able to accumulate a broad range of metal ions via "bioaccumulation" mechanisms. With certain metals, adventitious uptake may occur because the transported metals are similar to essential elements needed for cell functioning, so are actively taken up into the cell. Uranium has no known biological function, and it has been suggested that uranium may be taken up into cells due to increased membrane permeability, caused for example by uranium toxicity (Suzuki and Banfield, 1999). Almost all published observations of intracellular uranium have been of uranyl phosphates in *Pseudomonas* species (Kazy et al., 2009; VanEngelen et al., 2010; Choudhary and Sar, 2011), although one study identified uranium bioaccumulation in an environmental isolate closely related to *Arthrobacter ilicis* (Suzuki and Banfield, 2004). Although of academic interest, there is scant evidence suggesting bioaccumulation of uranium would be a viable technique for bioremediating contaminated land or water.

2.4. Biosorption

Biosorption describes the passive uptake of uranium to the surface of living or dead microbial cells. Both Gram-positive and Gram-negative bacterial cell envelopes possess an electronegative charge, so are able to attract metal cations which sorb to the surface. Ligands in the cell wall such as carboxyl, amine, hydroxyl, phosphate and sulfhydryl groups bind metals through chemical sorption (Beveridge and Murray, 1980; Lloyd and Macaskie, 2000). Biosorption is perhaps best suited to treating effluents with low to medium metal concentrations because binding to cell walls is faster than uptake into the cell, and it is easier to remove bound metals from a cell surface to regenerate the biosorbent (Schiewer and Volesky, 2000). Dead biomass is often a better biosorbent as the effects of metal toxicity are not important. A review of microbial biosorption capacity found uranium uptake in bacteria ranged from 45 to 615 mg g^{-1} cell dry weight (Suzuki and Banfield, 1999).

Despite the potential for bacteria to biosorb uranium, it is unlikely to be useful in the context of bioremediation. Problems associated with biosorption are that desorption from cell surfaces can be as rapid as sorption, and other cations compete for binding sites (Schiewer and Volesky, 2000). Cell surfaces can also quickly become saturated, preventing further biosorption. Sorbed material could be re-released to solution when cells die and decompose, although in one study, simulated cell decomposition facilitated the precipitation of uranyl phosphate (Knopp et al., 2003). Furthermore, a critical review of biosorption noted that regardless of the significant amounts of research, there has been almost no industrial application of biosorption (Gadd, 2009). These challenges mean that it is not an adequate long-term solution for in situ bioremediation, although it could be potentially used for treating contaminated effluent in a "pump and treat" scenario.

3. Uranium bioreduction

3.1. Early work & mechanisms

Bacteria capable of completely oxidising organic matter coupled to the reduction of Fe(III) or Mn(IV) were first described by Lovley and Phillips (1988) and Myers and Nealson (1988). An environmental isolate from freshwater sediments (later designated *Geobacter metallireducens*) was able to enzymatically reduce Fe(III) as ferrihydrite gel to magnetite or vivianite while oxidising acetate to CO_2, obtaining energy for growth. When exposed to U(VI), the cells reduced it to a poorly soluble U(IV) phase, and were able to grow in an appropriate medium, until U(VI) became depleted (Lovley et al., 1991). In parallel, another bacterium isolated from freshwater sediments (*Alteromonas putrefaciens* strain MR1, later designated *Shewanella oneidensis* MR1 (Venkateswaran et al., 1999)) was also found to be able to couple growth to the reduction of Mn(IV) and Fe(III) (Myers and Nealson, 1988; Lovley et al., 1989) and U(VI) (Lovley et al., 1991).

A relatively wide diversity of prokaryotes has been shown to enzymatically reduce U(VI) (Williams et al., 2012). As well as *Geobacter* and *Shewanella* species, dissimilatory U(VI) reduction has been identified in the sulfate-reducers *Desulfovibrio desulfuricans* and *Desulfovibrio vulgaris*, which produced the U(IV) mineral uraninite via *c*-type cytochrome activity (Lovley and Phillips, 1992a, 1992b; Lovley et al., 1993a). Other species identified to enzymatically bioreduce U(VI) include the sulfate-reducer *Desulfosporosinus* (Suzuki et al., 2002, 2003), *Anaeromyxobacter* species (Sanford et al., 2007), *Paenibacillus* (Ahmed et al., 2012a), *C. ferrireducens* (Khijniak et al., 2005), and Gram-positive *Clostridium* species (Francis et al., 1994; Suzuki et al., 2003; Madden et al., 2007) and *Cellulomonas* species (Sani et al., 2002; Sivaswamy et al., 2011). Additional genera listed in a literature review on the subject

include *Deinococcus*, *Desulfomicrobium*, *Desulfotomaculum*, *Pseudomonas*, *Pyrobaculum*, *Salmonella*, *Veillonella*, *Thermoanaerobacter* and *Thermus* (Wall and Krumholz, 2006). Recently it has been recognised that as well as vegetative cells, spores are able to facilitate U(VI) reduction suggesting a microbial pathway for U(VI) reduction in more extreme environments (Junier et al., 2009; Dalla Vecchia et al., 2010). Not all these bacteria can gain sufficient energy for growth from U(VI) respiration. Those which are known to conserve energy using U(VI) as the sole electron acceptor include: *S. oneidensis*, *G. metallireducens*, *G. lovleyi*, *G. sulfurreducens*, *Desulfotomaculum reducens*, *C. ferrireducens* and *Anaeromyxobacter dehalogenans* (Lovley et al., 1991; Tebo and Obraztsova, 1998; Khijniak et al., 2005; Wall and Krumholz, 2006; Sanford et al., 2007).

The mechanism by which cells transfer electrons from the electron donor to an electron acceptor such as U(VI) is debated, and it is thought that different mechanisms may exist for different species. For example, the consensus is *Shewanella* species do not necessarily require direct contact with the electron acceptor, while *Geobacter* species do. Although U(VI) can be significantly soluble in certain environmental conditions and therefore may diffuse into direct contact with a cell, bacteria are also capable of transferring electrons to solid electron acceptors such as Fe(III) or sorbed/precipitated U(VI). Therefore bacteria must have evolved mechanisms for transporting electrons from the central metabolism to the outside of the cytoplasmic membrane, the periplasm, the outer membrane (of Gram-negative cells), and potentially extracellularly. These include the use of electron-carriers such as cytochromes or flavins, or through the expression of conductive cell surface appendages such as pili. Indeed, extracellular electron transfer has been observed on the scale of centimetres in marine sediments, in addition to the usual nanometre scale (Nielsen et al., 2010; Pfeffer et al., 2012). The mechanisms of U(VI) bioreduction are not yet fully resolved, especially the significance of the role played by pili and electron shuttles. Reduction of U(VI) to U(IV) requires two electrons to be transferred, however, it has not yet been demonstrated whether bacteria are able to do this directly. One study using *Geobacter sulfurreducens* identified that U(VI) was reduced to the unstable intermediate U(V), which then disproportionated to the end product, U(IV) (Renshaw et al., 2005).

3.1.1. Cytochromes

The *c*-type cytochromes are essential proteins used by *Geobacter* and *Shewanella* to transfer electrons from the cytoplasmic membrane to the outer membrane (Lovley et al., 1993a; Richter et al., 2012). In *Shewanella,* the association of *c*-type cytochromes with extracellular polymeric substance containing biogenic uraninite has been demonstrated (Marshall et al., 2006). The cytochrome c_3 was identified as the U(VI) reductase in *Desulfovibrio vulgaris* (Lovley et al., 1993b).

Multiple lines of evidence suggest that *c*-type cytochromes contribute to U(VI) reduction in *Shewanella*, including observations of changes in cell cytochrome content, experiments with mutants lacking in certain cytochromes, and genomic sequencing (Wall and Krumholz, 2006). Mutant studies with *Shewanella* have found that while cytochromes, quinones and structural proteins are all needed for optimal U(VI) reduction, they are not essential, which indicates multiple pathways for electron transport.

c-Type cytochromes play an important role in U(VI) reduction by *Geobacter sulfurreducens*. Experiments with mutant strains suggest both periplasmic and outer membrane cytochromes are potentially involved. The periplasmic *c*-type cytochrome PpcA was identified to participate as an intermediary electron carrier during electron transfer from acetate to U(VI) (Lloyd et al., 2003). These results were not replicated by a later study, perhaps due to a modified methodology being used (Shelobolina et al., 2007). Instead, removing outer membrane cytochrome activity was found to have a greater effect on the rate of U(VI) reduction. One periplasmic cytochrome, MacA, was observed to be significant in reducing U(VI). As these outer membrane cytochromes were not able to reduce U(VI) directly, the authors proposed that U(VI) reduction occurred at the cell surface. Another study with mutant strains of *Geobacter sulfurreducens* found that in order to substantially lower the rate of U(VI) reduction compared to wild type, the genes for the five most abundant *c*-type cytochromes had to be deleted (Orellana et al., 2013). This suggests that that a diverse range of outer surface cytochromes can participate in U(VI) reduction, consistent with results for other extracellular electron acceptors.

Interestingly, recent work suggests that Gram-positive bacteria may use cytochromes to reduce Fe(III) (Carlson et al., 2012; Gavrilov et al., 2012), and this may have relevance to U(VI)- and radionuclide-reduction such as at alkaline pH where Gram-positive bacteria have also been implicated in metal reduction processes (Khijniak et al., 2005; Williamson et al., 2013).

The ability of particular proteins to reduce U(VI) is described as being fortuitous; the evolution of specific U(VI) respiratory pathways is considered unlikely given the low uranium content of natural groundwaters (Lovley, 2011; Cason et al., 2012; Williams et al., 2012). This non-specific protein activity is thought to be widespread in microbial U(VI) reduction, especially as in a similar way to with humic substances, a diverse range of *c*-type cytochromes are able to transfer electrons to U(VI) in *Geobacter* species.

3.1.2. Nanowires

The ability to express pili (nanowires) and flagella on one side of the cell has been observed in *Geobacter* species (Childers et al., 2002). Flagella are required for mobility, and are suggested to be a more energy efficient method of reaching an electron acceptor compared to, for example, the use of electron shuttles. *Geobacter* pili were found to be highly conductive, and so were proposed to act as a conduit for electrons from the cell to the surface of iron oxides (Reguera et al., 2005). A pili-deficient mutant was unable to reduce Fe(III)-oxides but was able to reduce soluble Fe(III) citrate, highlighting the potential importance of pili in extracellular electron transport in some systems. The *c*-type cytochrome OmcS, located on the pili of *Geobacter*, is thought to be required to transfer electrons between the cell and Fe(III)-oxides (Leang et al., 2010; Lovley, 2011; Mehta et al., 2005). Similar results occurred with U(VI) and furthermore, U(IV) was observed to precipitate along the pili, preventing periplasmic mineralisation and so preserving cell viability (Cologgi et al., 2011). The rate and extent of U(VI) reduction was greater when pili were expressed. However, these results were not replicated in a later study, with the pili-deficient mutant precipitating only slightly less U(IV) than the wild type strain (Orellana et al., 2013). Electron microscopy imaging of the wild type strains revealed U(IV) was not precipitated along the pili, instead it was mainly located at the outer membrane. Furthermore, a mutant with normal outer surface *c*-type cytochrome activity which produced low conductivity pili was able to reduce U(VI) at rates only slightly lower than wild type, challenging the importance of electron transfer through pili for U(VI) reduction. The precise mechanism of electron transfer to U(VI) in these systems, especially regarding the involvement of pili, remain hotly debated (Williams et al, 2012).

3.1.3. Extracellular electron carriers

To respire insoluble Fe(III) oxides, *Shewanella* can release chelators to solubilise Fe(III) and/or electron shuttles to mediate extracellular electron transfer (Lovley et al., 2004). Release of flavin mononucleotide and riboflavin by *S. oneidensis* MR-1 is an important process in transferring electrons to poorly soluble Fe(III) oxides (Marsili et al., 2008; von Canstein et al., 2008), with active secretion rather than release through cell lysis the dominant mechanism (Brutinel and Gralnick, 2012). Flavin mononucleotide has also been shown to mediate the reduction of U(VI) to U(IV) by *Shewanella* (Suzuki et al., 2010).

3.2. Mineralogical endpoints of bioreduction

Early uranium bioreduction experiments used X-ray diffraction (XRD) and transmission electron microscopy (TEM) to identify the

black mineral precipitate formed as uraninite $UO_{2(c)}$ (Lovley and Phillips, 1992a; Abdelouas et al., 1998). Nanometre sized particles of uraninite have been identified using high resolution TEM and X-ray Absorption Spectroscopy (XAS) (Suzuki et al., 2002, 2003; Burgos et al., 2008; Schofield et al., 2008; Jiang et al., 2011). More recently, another form of U(IV) has been identified using XAS (Kelly et al., 2008; Bernier-Latmani et al., 2010; Fletcher et al., 2010; Boyanov et al., 2011; Cologgi et al., 2011; Sharp et al., 2011; Latta et al., 2012). This non-crystalline disordered U(IV) phase, coordinated with carboxyl or phosphate ligands is commonly termed "monomeric" U(IV) (Bernier-Latmani et al., 2010). Determining whether U(VI) is reduced to uraninite or monomeric U(IV) is of interest to long-term remediation strategies; both are susceptible to reoxidation but uraninite has been suggested to be less prone to reoxidation due to its crystalline structure. However, a recent study comparing the susceptibility of bioreduced U(IV) as biogenic uraninite and monomeric U(IV) found little difference in their oxidation rates under controlled experimental conditions (Cerrato et al., 2013). Other U(IV) minerals include coffinite [$USiO_4 \cdot nH_2O$] and ningyoite [$CaU(PO_4)_2 \cdot 2H_2O$]; these are less susceptible than uraninite to remobilisation but coffinite has never been identified as the end-product of uranium bioreduction although ningyoite has occasionally (Khijniak et al., 2005; Lee et al., 2010).

Review of this work indicates that uraninite is produced in experiments using bacterial pure cultures conducted in a simple medium and is precipitated within the periplasm, on the cell surface or extracellularly (Lloyd et al., 2002; Suzuki et al., 2002). Monomeric U(IV) tends to be produced in experiments using bacterial pure cultures in complex media (Bernier-Latmani et al., 2010; Alessi et al., 2012; Cerrato et al., 2013), when phosphate is added (Boyanov et al., 2011), or, under certain conditions, when natural sediments are included (Kelly et al., 2008, 2009; Sharp et al., 2011; Alessi et al., 2012). Ageing of U minerals to more crystalline forms has been observed, such as from monomeric U(IV) to uraninite (Kelly et al., 2009). However, a long-term study using *Thermoanaerobacter* to bioreduce a mixture of U(VI) and FeOOH found nanocrystals of uraninite present after three months incubation persisted for three to four years suggesting no evidence for ageing and increasing crystallinity (Madden et al., 2012). Furthermore, analysis of in situ sediment columns found no evidence of transformation of monomeric U(IV) to uraninite; similar abundances of monomeric U(IV) and uraninite were observed post-U(VI) bioreduction and after one year of in-well ageing (Bargar et al., 2013).

Cell wall architecture has been suggested to influence the form of bioreduced U(IV) as under the same conditions (with no phosphate present), different U(IV) end products were generated by Gram-negative *Anaeromyxobacter* and Gram-positive *Desulfitobacterium* (Boyanov et al., 2011). The authors reason that uranyl carbonate complexes are reduced to U(IV) complexed to carbonate, and that neutral or positively charged uranyl complexes are reduced to free U(IV) which can form uraninite. It was proposed that outer membrane reductases in Gram-negative bacteria allow direct electron transfer to sorbed positive or neutral uranyl complexes, but as these are most likely lacking in Gram-positive bacteria, they perhaps rely on soluble mediators to reduce negatively charged aqueous uranyl carbonate complexes instead. However, in contrast to this theory, direct evidence for cell wall cytochrome participation in Fe(III) reduction was observed using Gram-positive *Thermincola potens* strain JR (Carlson et al., 2012). Furthermore, direct contact between Gram-positive *C. ferrireducens* and ferrihydrite was required for Fe(III)-reduction; no evidence was observed for electron shuttles or chelators, and the use of a cytochrome inhibitor indicated that cytochrome-bc1-complex was pivotal in ferrihydrite reduction (Gavrilov et al., 2012). Further work to elucidate the mechanism(s) of uranium reduction in Gram-positive bacteria is clearly warranted as they appear to be relevant to high pH conditions (Williamson et al., 2013).

3.3. Field studies

In situ bioreduction of U(VI) in the field (Fig. 3) has been demonstrated successfully at pilot scale e.g. Anderson et al. (2003), Istok et al. (2004), Williams et al. (2011), although maintaining low U(VI) concentrations in groundwater over long periods of time may require a repeated supply of electron donor. Numerous factors determine whether bioreduction will be successful or not, from the presence of a suitable electron donor, to competition from other processes such as nitrate and sulfate reduction. Environmental conditions will also control the composition of the microbial community and population dynamics (Williams et al., 2012). The long-term stability of the mineral phases formed is crucial to the success of in situ bioremediation; the more insoluble a mineral is, the less likely it will be remobilised. It is also important to avoid clogging of the injection well and aquifer through biomass growth or excess mineral precipitation, and to consider dilution effects from pumping large volumes of water and electron donor.

While bacteria are generally thought of as being radiotolerant, too high concentrations of U(VI) will have an inhibitory effect, either through radiotoxicity or, more likely for normal isotopic compositions, chemotoxicity. Experiments with enrichment cultures from the Oak Ridge site found that the inhibition co-efficient for U(VI) was around 100 μM; at this level the effective yield and growth rate were reduced by 50% (Nyman et al., 2007). Note that while this concentration of U(VI) far exceeds the concentrations reported in groundwater in the vicinity of the S3 ponds, of up to 11 μM (Spain and Krumholz, 2011), in other areas of the site concentrations greater than 100 μM have been reported, such as 250 μM in well FW113-47 (Cho et al., 2012).

Bacteria can use a wide range of organic carbon sources as electron donors. Determining which is most efficiently coupled to U(VI) reduction is an important step in tailoring bioremediation strategies for different sites. Acetate is the most commonly used electron donor in laboratory and field experiments, followed by ethanol and lactate. An alternative approach is to use electrodes to donate electrons for U(VI) reduction (Lovley and Nevin, 2011). Studies comparing electron donors suggest that the most effective donor is specific to an individual site, for example, ethanol was recommended for the Oak Ridge site (Luo et al., 2007) while acetate was for the US DOE Shiprock site (Finneran et al., 2002a). Column experiments using alluvial Rifle sediments found that while a time lag was observed when hydrogen release compounds (HRC) and vegetable oil were used, the extent of U(VI) removal was greater with these donors compared to acetate (Barlett et al., 2012a). Rates of U(VI) reduction in Oak Ridge sediments using ethanol, glucose, methanol and methanol with added humic acids were nearly equivalent when donor concentrations were normalised for equivalent electron donor potential yield (Madden et al., 2009). A column study with Oak Ridge sediments found that acetate and lactate showed similar trends in U(VI) reduction, with comparable amounts removed over approximately one year (Tokunaga et al., 2008). An intermediate electron donor supply rate achieved optimal U(VI) reduction; too low rates were insufficient to stimulate bioreduction, while soluble uranyl carbonate complexes formed from oxidation of the organic carbon at the lower and higher supply rates of organic carbon supply. Clearly the selection and application of an electron donor needs to be carefully considered on a site-specific basis. Genomic modelling has recently been developed to predict the response of microbial communities to bioremediation (Williams et al., 2012). The Bottom-Up Genome Scale (BUGS) approach has been used to successfully to predict competition for electron donor between species capable of U(VI) reduction and those unable to reduce U(VI), but able to couple electron donor oxidation to Fe(III) or sulfate reduction (Barlett et al., 2012b; Zhuang et al., 2012). Further development of this model will allow application of bioremediation to be customised to site-specific microbial communities (Williams et al., 2012).

As well as being able to drive the biotic reoxidation of biogenic U(IV), nitrate also acts as a competing electron acceptor. A number

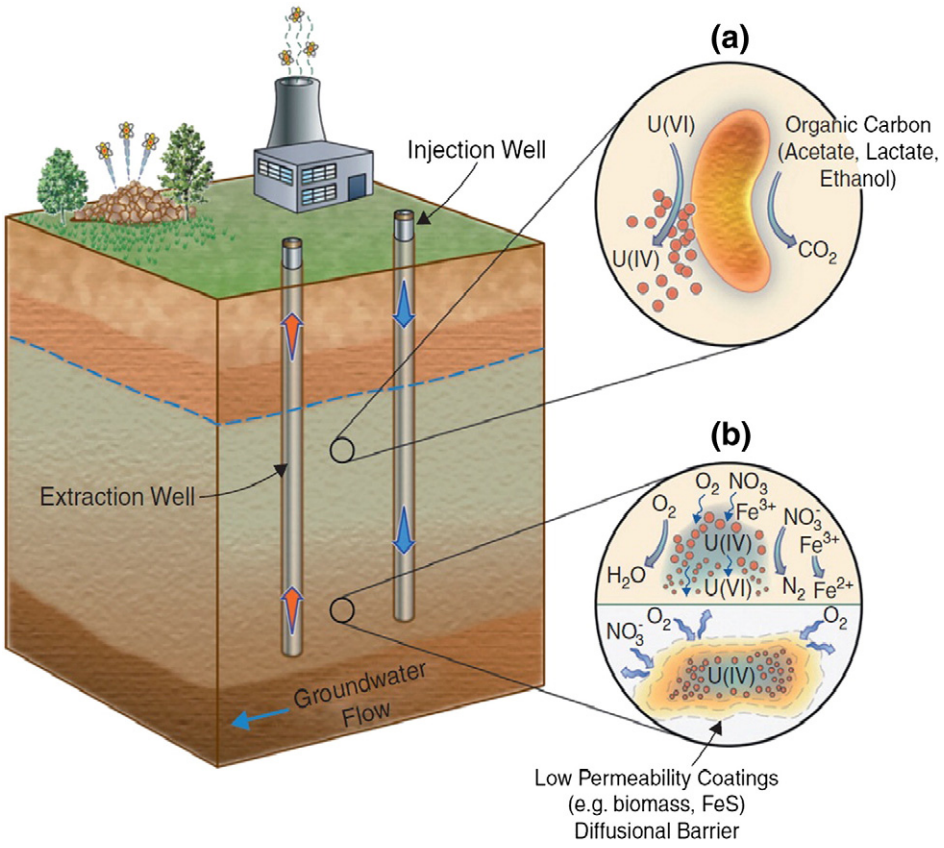

Fig. 3. Conceptual illustration of the process of uranium bioremediation after Williams et al. (2012). (a) Indigenous microorganisms present in soils, sediments, and groundwater contaminated by nuclear energy and weapons production activities are stimulated through introduction of organic carbon compounds via injection wells. Select organisms may couple the oxidation of organic carbon (and H_2) to the reduction of aqueous uranium, as U(VI), converting it from a soluble to an insoluble form, as U(IV). (b) Reduced U(IV) may be re-oxidised to U(VI) following cessation of organic carbon injection accompanying subsequent delivery of oxidants, such as O_2, NO_3, and Fe^{3+}; the presence of diffusional barriers (e.g., biomass or low permeability sediments) or preferential reductants (e.g., FeS) can suppress re-oxidation and maintain stability of immobilised U(IV).

of studies have shown that nitrate is preferentially used as an electron acceptor before U(VI) and Fe(III) due to it being more energetically favourable (DiChristina, 1992; Finneran et al., 2002b; Istok et al., 2004), although concomitant U(VI) and nitrate reduction has been demonstrated (Madden et al., 2007). As nitrate is a common co-contaminant with uranium at nuclear sites, this is a potential impediment to the application of bioreduction as a remediation strategy. An alternative theory is that the presence of nitrate is beneficial under low pH conditions, because consequent denitrification produces OH^- and HCO_3^-, neutralising the pH and thus stimulating metal reduction (Law et al., 2010a; Thorpe et al., 2012a).

U(VI) forms stable complexes with carbonate in natural waters under oxic conditions and at pH > 6.5 (Langmuir, 1978). High concentrations of carbonate can stabilise U(VI) in solution therefore preventing it from being immobilised. Calcium-uranyl-carbonate complexes are very stable; in this form U(VI) is a much less favourable electron acceptor (Brooks et al., 2003). Indeed, higher concentrations of bicarbonate (40 mM) were found to lower the rate of U(VI) reduction in contaminated sediments (Luo et al., 2007) and the presence of 0.45–5 mM calcium significantly reduced the rate and extent of U(VI) bioreduction by *Shewanella*, *Desulfovibrio* and *Geobacter* (Brooks et al., 2003; Stewart et al., 2011). In contrast, results of bioreduction experiments with U(VI) sorbed to sediments representative of the Dounraey nuclear facility found reduction and immobilisation occurred even in the presence of micromolar quantities of bicarbonate and calcium (Begg et al., 2011). Similarly, experiments at the Rifle site with 5 mM Ca and abundant bicarbonate still show rapid rates of U(VI) bioreduction in situ (Williams et al., 2011).

3.3.1. US DOE Rifle site, Colorado

Groundwaters at this former uranium ore processing facility are contaminated with low levels of uranium, which leached from mill tailings into the unconfined aquifer (Anderson et al., 2003). The aquifer is an alluvial deposit of the nearby Colorado River, and groundwater flows from the site into the river at around 0.8 m per day. Vertical migration of uranium-contaminated groundwater is limited by the Wasatch formation; a silty shale which acts as an aquitard (Zachara et al., 2013). Evidence of deposition of uranium as U(IV) has been observed in naturally reducing zones of the aquifer; XAS analysis of one sample particularly high in natural uranium identified monomeric U(IV) complexed to organic matter, Fe(II) and sulfide (Campbell et al., 2012). Release of contaminant U(IV) from naturally reduced zones by oxidation, together with migration of up-gradient groundwater naturally high in U(VI) are responsible for the persistence of elevated concentrations of uranium in groundwater, despite the mill tailings being removed from site during the 1990s (Zachara et al., 2013).

Biostimulation with an injectate of 100 mM acetate was trialled at the site in 2002 (Anderson et al., 2003). Groundwater from an up-gradient well was collected and amended with the electron donor and 10 mM Br^- as a conservative tracer, before being injected into the treatment area over a three month period to generate 1 to 3 mM acetate in situ. Bromide detection demonstrated an average 2% volume addition to the aquifer per day. Within 50 days, U(VI) concentrations decreased from between 0.4 and 1.4 μM to below the maximum contaminant limit of 0.18 μM, with concurrent release of Fe(II). After 50 days, U(VI) concentrations began to increase and Fe(II) decreased. At the same time, sulfate decreased stoichiometrically with acetate consumption suggesting a release of U during early sulfate reduction.

Field trials also took place at the Rifle site during 2007 and 2008 (Williams et al., 2011). Even though more than 90% of U(VI) was present as recalcitrant uranyl-calcium-carbonate complexes, when acetate was supplied as an electron donor, concentrations of U decreased from 1–1.5 μM to 0.05–0.1 μM. These low concentrations were maintained over long periods (>140 days) by ensuring the concentration of acetate remained greater than the 10 mM sulfate present. A shorter period of acetate amendment (at 5 mM) during the first trial caused a temporary increase in U(VI) at the onset of sulfate reduction. This was attributed to the increasing alkalinity and pH which promoted U(VI) desorption and complexation with carbonate. Another factor might be that sulfate-reducing bacteria were not able to couple U(VI) reduction to acetate oxidation at Rifle; perhaps unsurprisingly given most sulfate-reducing bacteria studied to date have used lactate as an electron donor for U(VI) reduction. However, when the system was not limited by acetate availability (supplied at 15 mM), concomitant Fe(III)- and sulfate-reduction occurred leading to accumulation of iron sulfides in soils and sustained U(VI) removal. Prolonged Fe(III)-reduction and sequestration in iron sulfides may prevent abiotic reoxidation of U(IV) by Fe(III) phases, while maintaining redox conditions under which U(IV) is stable. Once acetate amendment was stopped, U(VI) concentrations rebounded, although U(VI) levels remained 30–55% lower than pre-injection levels for more than 210 days in wells which had received prolonged acetate delivery. Stable isotope probing and gene expression analysis confirmed *Geobacter* were active and oxidising acetate, even during sulfate reduction, so are likely to be responsible for U(VI)-reduction and maintaining low concentrations of U(VI) in groundwater. During this trial the authors reported a decrease in hydraulic conductivity of four orders of magnitude at the injection well, possibly due to precipitation of carbonate and sulfide minerals and biomass accumulation, but this did not impede electron donor delivery and was not observed at any monitoring wells.

Monitoring of uranium isotope ratios during field trials found $^{238}U/^{235}U$ in groundwater decreased significantly during in situ bioreduction (Bopp et al., 2010). This is the opposite of what was expected, as generally lighter isotopes react faster than heavier, although it can be explained by an effect known as “nuclear field shift”. A bicarbonate injection designed to induce uranium desorption caused no change in the isotopic ratio, clarifying that adsorption and desorption do not impact $^{238}U/^{235}U$ and therefore uranium isotope ratios may be used to indicate the occurrence of in situ bioreduction (Shiel et al., 2013). A number of geophysical techniques have been used to monitor the effects of in situ biostimulation trials including measurement of: spectral ionisation potentials (Williams et al., 2009), self potentials (Williams et al., 2010a), current density (Williams et al., 2010b) and complex resistivity (Orozco et al., 2011). As geophysical techniques can cover larger areas and offer continuous time coverage compared to conventional geochemical analyses from borehole samples, they may considerably improve understanding of the changes occurring in the subsurface during biostimulation. Furthermore they could be used to provide real-time information to optimise biostimulation, such as allowing the acetate injection rate to be adjusted in order to maintain metal-reducing conditions (Orozco et al., 2011).

In situ sediment columns were deployed during a subsequent 2009 acetate-amendment field trial (Bargar et al., 2013). Most of the U(VI) was reduced during sulfate-reducing conditions and a close association was observed with U(IV) and Fe-sulfide (mackinawite) coatings on sediment grains, although this was heterogeneous at the micrometre and sub-micrometre scales. Mackinawite is known to be able to reduce U(VI) to uraninite abiotically (Hyun et al., 2012) when phosphate concentrations are low, such as in Rifle groundwater. Two forms of U(IV) were identified; uraninite and monomeric U(IV) associated with biomass-derived phosphoryl ligands. The authors proposed that the juxtaposition of biomass and mackinawite allows for the concurrent deposition of monomeric U(IV) and uraninite via a biotic–abiotic transition pathway. Moreover, the simultaneous precipitation of U(IV) phases with sulfides creates physical and chemical barriers to U(IV) re-oxidation, potentially explaining how U(VI) removal is maintained post-acetate amendment.

The microbial communities stimulated at Rifle have been studied in detail using state-of-the-art molecular analyses. The PhyloChip microarray identified background Rifle sediments to contain diverse microbial communities (Handley et al., 2012). In general, *Geobacter* species have been found to dominate the microbial community during U(VI) bioreduction (Anderson et al., 2003; Chang et al., 2005; Chandler et al., 2010). Use of ^{13}C labelled acetate found a relatively diverse active microbial community prior to acetate addition, but *Geobacter*-like species dominated at the end of the trial (Kerkhof et al., 2011). Of the total *Geobacter* population, 90% of the cells were planktonic during the peak phase of Fe(III)-reduction, whereas 77% were attached to sediment surfaces during sulfate-reduction, as were 75% of sulfate-reducing bacteria (Dar et al., 2013). The authors suggest this is likely due to *Geobacter* having more energy to be motile and being able to seek out Fe(III) during periods of excess electron donor availability. Whole genome microarray analyses found the transcript abundance of *rpsC* (ribosomal proteins S3) correlated best with the growth rate of *Geobacter uraniireducens*, therefore monitoring expression of *rpsC* could be used to measure *Geobacter* metabolism during biostimulation (Holmes et al., 2013a). Phospholipid fatty acid analysis (PLFA) identified a large increase in biomarkers for *Geobacter* species and an unidentified Fe(III)-reducer during an acetate biostimulation field trial (Peacock et al., 2011). Proteomic analysis of planktonic biomass dominated by *Geobacter* detected an abundance of enzymes and peptides associated with acetate metabolism and energy generation (Wilkins et al., 2009). These data were used to validate an in silico genome-scale model of *G. metallireducens*, which may be used in future to manipulate geochemical conditions during uranium bioreduction, and so achieve cost effective bioremediation (Fang et al., 2012). The overall species diversity was lower in samples which had been biostimulated with acetate, but there was an increase in Fe(III)-reducing and sulfur redox cycling genera, especially organisms affiliated with the *Desulfuromonadales* and *Desulfobacterales* (Handley et al., 2012). A shift from Fe(III)-reducers to sulfate-reducers was observed as the trials progressed (N'Guessan et al., 2008). Analysis using the GeoChip microarray identified a change in microbial functional gene abundance, from genes predominantly used for metal reduction e.g. *c*-type cytochromes, to genes required for sulfate reduction and methane generation (Liang et al., 2012). A similar shift was detected using proteomic techniques (Callister et al., 2010). Post-trial, members of the *Firmicutes* group closely related to *Mollicutes* and *Clostridia* dominated, although these were thought to remove U(VI) via adsorption rather bioreduction (N'Guessan et al., 2008). Proteomic analysis identified a legacy effect on the microbial community caused by field trials; a more diverse community remained following the 2007 trial which may have impacted the 2008 trial by decreasing the duration of Fe(III)-reduction (Callister et al., 2010). Finally, analysis of 18S rRNA gene sequences during an acetate amendment field trial revealed a predator–prey response between bacteriovorous protozoa and metal- and sulfate-reducing bacteria (Holmes et al., 2013b). An initial bloom of *Geobacter* was followed by an increase in a species closely related to *Breviata anthema*, an ameboid flagellate, while diplomonadid flagellates from *Hexamitidae* accompanied the bloom of sulfate-reducing *Peptococcaceae*. Although largely unexplored, predator–prey relationships may play an important role in subsurface microbial ecology, and may limit the rate of U(VI) reduction.

Finally, reactive transport modelling (RTM) has been used to simulate the in situ biostimulation trials at Rifle. In brief, RTM computes contaminant transport using:

- hydrogeological parameters to describe groundwater flow, obtained by monitoring transport of an inert tracer such as bromide; and

- geochemical parameters to estimate chemical reactions that may aid or hinder contaminant transport — this requires knowledge of, amongst other things, the microbial community and their metabolic pathways, and the likelihood of sorption.

The first application of RTM to Rifle was for the 2002 biostimulation trial (Yabusaki et al., 2007). Data on the injection tank composition and drawdown, together with field bromide tracer data were used to define groundwater transport. Equations governing the coupling of Fe(III) oxyhydroxides and U(VI) as electron acceptors to the consumption of acetate by *Geobacter* and sulfate-reducers, were used to represent the geochemical component of the model. These parameters were adjusted to reflect field geochemical data from the 2002 trial for both stimulated and background wells. Subsequently this RTM was successfully applied to a 2003 trial (in the same boreholes) without parameter modification, highlighting the usefulness of RTM to predict future contaminant transport. Additional development of the model to incorporate uranium adsorption and various mineral reactions, again benchmarked with the 2002 hydrogeological/geochemical parameters, was found to be applicable to a 2007 biostimulation trial in a different plot within the Rifle site (Fang et al., 2009). Recent developments in RTM of Rifle biostimulation trials include incorporation of microbial growth equations coupled to abiotic geochemical reactions (Istok et al., 2010), three-dimensional variably saturated flow (Yabusaki et al., 2011) and proteomic data (Fang et al., 2012). In parallel, RTM has been used to consider the effect of biomass growth and mineral precipitation during biostimulation on groundwater flow. Geochemical data from column experiments were used in a reactive transport model to predict accumulation of minerals and biomass for the 2002 and 2003 Rifle biostimulation trials; results suggested clogging of pore space may occur in the vicinity of electron donor injection wells (Li et al., 2009). Subsequent modelling confirmed this, and also emphasised the influence of physical and geochemical heterogeneities on the spatial distribution of pore clogging and its effect on hydraulic conductivity (Li et al., 2010, 2011).

In summary, a number of field trials at the US DOE Rifle site have demonstrated the potential for acetate application to stimulate bioreduction of U(VI) in groundwater and maintain low concentrations over long periods of time. It appears that *Geobacter* species play a major role in U(VI) reduction, and are active during Fe(III)- and sulfate-reducing conditions. Acetate amendment has a long-term effect on microbial community structure and diversity within the aquifer. State-of-the art molecular analysis and modelling continue to improve understanding of subsurface processes occurring during in situ biostimulation.

3.3.2. US DOE Oak Ridge site, Tennessee

This site was contaminated with uranium through disposal of wastes in unlined ponds between 1951 and 1983, including those from the cleaning of uranium processing equipment using nitric acid (Green et al., 2012). The groundwater in some areas of the site is therefore characterised by low pH, high nitrate and U(VI) contamination, posing difficult challenges for in situ bioremediation. Multiple migration pathways are present, causing distinct plumes with different chemical compositions. For example, in the vicinity of the S3 ponds, concentrations of uranium range from 0.015 to 10.9 μM and nitrate from 0.47 to 37 mM (Spain and Krumholz, 2011). Over 95% of the uranium in the Oak Ridge subsurface is bound to sediments (Wu et al., 2010).

Push–pull tests were conducted using ethanol, acetate or glucose as electron donors to assess the potential for bioreduction of U(VI), Tc(VII) and nitrate (Istok et al., 2004). Background concentrations in groundwater from test wells were 0–5.8 μM U, 0.039–18 nM Tc and 1–168 mM nitrate. The injection solution comprised site groundwater amended with 80–130 mM sodium bicarbonate, 1.3 mM bromide tracer and 20–200 mM of electron donor, pH adjusted using 80% N_2 20% CO_2. 200 l of injection solutions were pumped into each well over 0.5 to 2 days, and the wells monitored for up to approximately 40 days. In test wells, dilution-adjusted concentrations of Tc(VII) and nitrate decreased, nitrite was produced, but no reduction of Fe(III), U(VI) or sulfate was detected. The only changes observed in control wells were decreases in concentration due to dilution. A second identical injection generated Fe(III)-reducing conditions and increased the reduction rate of nitrate and Tc(VII) and stimulated limited reduction of U(VI).

A pilot field experiment was set up in which groundwater was firstly pre-treated to condition it prior to adding an electron donor to bioreduce uranium (Wu et al., 2006a, 2006b). The treatment area was selected for high hydraulic conductivity and relatively high uranium concentrations. Purged site groundwater was treated above ground by adjusting the pH to 4.3–4.5 to remove aluminium and calcium which otherwise might cause in situ clogging of the aquifer, and also to remove nitrate. This treated water was supplemented with tap water and returned to ground. Subsequently the pH of the treated water was increased to 6.0–6.3, in order to increase the subsurface pH to generate optimal conditions for microbial activity. Unsurprisingly these flushing phases drastically reduced the concentrations of contaminants in the groundwater, from an initial 48–158 μM U to 2.7–5.1 μM, and from 114–271 mM nitrate to 0.1–0.78 mM. Uranium in soils remained around 800 mg/kg. Following conditioning, ethanol was added intermittently to stimulate bioreduction. Nitrate reduction occurred for the first 47 days, followed by U(VI) reduction which decreased concentrations in groundwater to around 1 μM for the duration of the trial (350 days). Final uranium concentrations in soil ranged from 910 mg/kg to 4320 mg/kg, with 28–51% present as U(IV); the highest values were found closest to the injection well.

A field trial comprising single injection of the slow-release electron donor emulsified vegetable oil (EVO) caused the concentration of U(VI) in groundwater to decrease from between 3.8 and 9.1 μM to less than 1 μM in each monitoring well tested (Gihring et al., 2011). U(VI) concentrations remained lower than the initial values for at least four to eight months. Later trials demonstrated a single application of EVO substantially reduced the mass of uranium discharged from the site to Bear Creek for more than a year before concentrations rebounded (Tang et al., 2013a, 2013b). Aqueous U(VI) concentrations initially increased as rates of U desorption attributed to biogenic bicarbonate production and Fe(III)-reduction exceeded U(VI) reduction. A biogeochemical model developed from initial laboratory experiments was used to simulate the field trial, and predicted substantial bioreduction and U(IV) accumulation, although at the time of writing this does not appear to have been confirmed with sediment analysis.

Nitrate-reducing bacteria form a high proportion of the microbial community sequences from background Oak Ridge sediments (Akob et al., 2007). The denitrifying bacterium *Rhodanobacter* dominated the acidic, nitrate-rich contaminated sediments (Green et al., 2012). Compared to pristine groundwater, water from contaminated wells had lower gene diversity but the signal intensity was higher (Waldron et al., 2009). Metal-resistant and metal-reducing microbes were present in both contaminated and pristine water, highlighting the potential for bioremediation. Known U(VI)-reducers *Desulfovibrio*, *Geobacter*, *Anaeromyxobacter*, *Desulfosporosinus* and *Acidovarar* species were detected in wells which had been biostimulated for nearly two years (Cardenas et al., 2008). Indeed, the presence *of Desulfovibrio*, *Anaeromyxobacter*, and *Desulfosporosinus* species as well as the abundance of *Geobacter* species could be used to indicate areas where U(VI) reduction had occurred (Cardenas et al., 2010). A recent study linked the transcript level of key functional genes with geochemical data on rates of bioreduction of Fe(III) and sulfate (Akob et al., 2012). The response of *Geobacteraceae*-specific *gltA* (which codes for an enzyme associated with integrating acetate in the TCA cycle) transcript levels were found to correlate with Fe(III) reduction activity, as did expression of the *drsA* gene (codes for the rate-limiting sulfate reduction enzyme) with sulfate concentrations. The type of electron donor used

can have a significant influence on the microbial community. Adding EVO as a slow-release electron donor initially caused members of *Veillonellaceae* and *Desulforegula* to dominate; these probably catalysed EVO decomposition and oxidised long-chain fatty acids to acetate (Gihring et al., 2011). An alternative approach to study the microbial community response is to use passive multilevel samplers deployed in situ into an electron donor injection well and down-gradient wells (Baldwin et al., 2008). Changes in community composition were observed to occur during biostimulation, with increases in cell density of denitrifying bacteria, delta-proteobacteria, *Geobacter* and methanogens.

In summary, most of the uranium in the Oak Ridge subsurface is bound to sediments. Multiples plumes of U(VI) in groundwater exist, some of which contain high concentrations of nitrate, which creates challenging conditions for U(VI) bioreduction. Application of EVO in situ to stimulate appears to be a promising bioremediation technique.

3.3.3. US DOE Hanford site, Washington

Uranium-containing liquid waste from fuel fabrication activity at the site was historically disposed of in trenches and ponds, which allowed leaching to groundwater (Peterson et al., 2008). A plume of uranium-contaminated groundwater is present with the unconfined aquifer of the alluvial Hanford formation; downward migration is limited by the consolidated fluvio-lacustrine Ringold formation (Zachara et al., 2013). Although the contaminant sources were removed in the 1990s, this plume persists with concentrations varying seasonally from 0.04 to 0.84 μM, in the form of very stable uranyl carbonate complexes (Peterson et al., 2008; Maher et al., 2012). The continued source of uranium is thought to be the release of adsorbed U(VI) from the vadose zone during spring groundwater rise driven by upstream snowmelt (Zachara et al., 2013). Under normal hydrological conditions, groundwater from the site discharges to the nearby Columbia River, while this reverses during periods of high river flow leading to a complex hydrodynamic regime and concentrations of uranium in river water between 2.1 and 7.1 nM.

The form of uranium in contaminated sediments has been investigated (Catalano et al., 2004, 2006). Sodium boltwoodite [$Na(UO_2)(SiO_3OH).1.5H_2O$] — a uranyl silicate from the uranophane group — predominated in the ground underlying a tank that was overfilled and leaked caustic aqueous sludge (of 2.5–5.0 M sodium carbonate with 0.5 M U(VI), 0.36 M phosphate and all fission products), presumably due to the high pH conditions in situ. Uranium was co-precipitated with calcite as micro-granules in the near surface underlying former process ponds, which had received wastes from the dissolution of nuclear fuel and cladding. Slightly deeper it was precipitated as metatorbernite [$Cu(UO_2PO_4)_2 \cdot 8H_2O$], while at depth it was sorbed onto phyllosilicates. Phylogenetic analysis of DNA from 21 Hanford sediment samples identified 1233 and 120 unique bacterial and archaeal operational taxonomic units respectively (Lin et al., 2012). Microbial diversity was greater in the oxic Hanford formation and lower in the deeper anoxic Ringold formation.

Column experiments using sediments from the site have demonstrated the potential for bioreduction to remediate U(VI) from groundwater (Ahmed et al., 2012b). These were set up supplied with oxic synthetic groundwater with or without organic amendment (2 mM lactate, 2 mM malate, 2 mM succinate and 2 mM fumarate), or deionised water, each amended with 0.126 mM U(VI) and monitored over 7 months. When synthetic groundwater amended with electron donors was used, 80 to 85% of U(VI) was immobilised via microbial reduction to uraninite. In the other columns, 100% of the U(VI) was adsorbed. Subsequent exposure to oxic Columbia River water over a 50 day period failed to remobilise more than 7% of U in each column.

Chemical remediation was trialled at the site over a five day period in 2007, aiming to immobilise uranium from groundwater into autunite [$(Ca,Mg,K,H)[(UO_2)(PO_4)]_{1-2}$] and apatite minerals [$Ca_5(PO_4)_3(OH,F,Cl)$] via phosphate injection (Vermeul et al., 2009). An earlier series of laboratory experiments identified a mixture of long-chain polyphosphates to be the most suitable for injection into ground (Wellman et al., 2007a,b, 2008). Uranium concentrations were initially reduced to below the maximum contaminant limit, however, within six weeks they had rebounded significantly. Uranium removal might have been due to the formation of autunite but it could have been due to flushing and dilution effects from the large volumes of water injected. The capacity for apatite formation under site conditions was suggested to be limited. This work illustrates the challenges of applying remediation in situ, namely the large volumes of water required to introduce sufficient phosphate, and the high concentrations of phosphate (10.5 mM) needed to remove relatively low concentrations of uranium (~1 μM). Furthermore, this trial reduced the hydraulic conductivity of the aquifer by on average a factor of six, over just five days. Moreover, despite demonstration in laboratory batch and column tests, the same results were not replicated under field conditions highlighting the complexities of scale-up.

In summary, despite the contaminant source being removed nearly 20 years ago, low concentrations of uranium persist in groundwater at Hanford. Column experiments demonstrated that uranium strongly sorbs to sediments under laboratory conditions; application of an electron donor did lead to bioreduction to uraninite but this was equivalent to sorbed U(VI) in terms of susceptibility to remobilisation. Developing a remediation strategy may be challenging given the difficulties in replicating site conditions in the laboratory, and the problems encountered during a trial of in situ chemical remediation.

3.4. Stability of bioreduced U(IV) and reoxidation

The resistance of poorly soluble U(IV) to reoxidation and consequent remobilisation as aqueous U(VI) is crucial for the success of remediation over the long term. Biogenic reduced nanoparticles have a large surface area so are more reactive (and potentially susceptible to reoxidation) than aggregates or crystals, although evidence suggests that biogenic uraninite nanoparticles aggregate, especially when formed by relatively slow rates of U(VI) reduction (Anderson et al., 2003; Senko et al., 2007). The presence of carbonate considerably increases the rate of uraninite reoxidation as it complexes with U(VI), removing it from the mineral surface and preventing a protective layer from accumulating (Ulrich et al., 2008; Campbell et al., 2011a). Thermodynamically, both oxygen and nitrate (via denitrification intermediates e.g. nitrite) should be able to reoxidise U(VI), but this may be limited by reaction kinetics. Here the focus is on reoxidation of U(IV) in sediments with natural microbial communities present, rather than on pure mineral forms. U(IV) may also be reoxidised by Fe(III) minerals (Sani et al., 2005; Ginder-Vogel et al., 2006; Spycher et al., 2011), manganese oxides (Fredrickson et al., 2002; Wang et al., 2013), organic ligands such as citrate and EDTA, even under anaerobic conditions (Luo and Gu, 2011), and microbially generated bicarbonate, even under bioreducing conditions (Wan et al., 2005, 2008).

3.4.1. Reoxidation by exposure to oxygen

The effects of reoxidation have been studied in terms of the long-term biocycling behaviour of radionuclides in natural and engineered environments. Laboratory microcosm experiments found near-complete reoxidation of U(IV) in sediment when gently agitated in air, within time periods as short as 24 h (Begg et al., 2011; Law et al., 2011). Around 60% of bioreduced Fe(II) was remobilised within 1 day (Burke et al., 2006) or 9 days (McBeth et al., 2007) after exposure to oxygen on an orbital shaker. Moderately fast reoxidation has been observed in column studies via purging the influent media with oxygen. For example, 61% of bioreduced U(IV) was remobilised within 21 days, and nearly all had been removed after 122 days (Komlos et al., 2008), while 88% of precipitated uranium was remobilised within 54 days of exposure to influent media containing 8.6 mg/l dissolved oxygen to represent the maximum concentration in groundwater at 15 °C (Moon et al., 2007). In contrast, negligible reoxidation of total bioreduced U(IV) was observed in a column supplied with oxygenated

influent for 64 days, although some modest localised reoxidation was observed (Sharp et al., 2011). The authors suggest this may be due to residual electron donor remaining in the sediments post-bioreduction.

Perhaps when trying to simulate the effects of oxic groundwater ingress a more environmentally-realistic oxidation method is to expose the sediments to naturally oxic water. A column study of this type used sediment and groundwater from the Rifle site, containing 1 to 2 mg/l dissolved oxygen (N'Guessan et al., 2010). Over the first month, 17% of the total U precipitated during the bioreduction phase was remobilised, after that no additional loss was detected. The microbial community became characterised by bacteria capable of oxidising complex organic matter from dead biomass, coupled to use of the low levels of dissolved oxygen present and this prevented reoxidation of biogenic U(IV). Experiments using sediments from the Hanford site found exposure to oxic river water over a 50 day period remobilised just 7% of bioreduced U(IV) in columns that had previously been supplied with an electron donor, and 7% of sorbed U(VI) in control columns (Ahmed et al., 2012b). The remaining 93% of U in the bioreduced column sediment was identified as nanoparticulate uraninite, suggesting it was recalcitrant to reoxidation under the conditions of study where, presumably, relatively low concentrations of dissolved oxygen were introduced to the column. Another environmentally relevant method used was to immerse biogenic uraninite into oxic groundwater within monitoring wells at the Rifle site (Campbell et al., 2011a, 2011b). After 104 days, approximately 50% had dissolved and no insoluble corrosion products were observed. This rate is 50 to 100 times slower than those measured in the laboratory, and this was attributed to the presence of biomass, molecular diffusion and surface passivation by groundwater solutes.

Reoxidation of bioreduced U(IV) has also been studied in situ at the Oak Ridge field site (Wu et al., 2007). Initial U(VI) concentrations in groundwater were up to 135 μM. Application of ethanol over a two year period stimulated immobilisation of uranium as U(IV). Subsequently sulfite was added to remove any remaining dissolved oxygen, which reduced concentrations of U(VI) in groundwater to <0.13 μM. Dissolved oxygen was introduced to the injection well over a 60 day period, causing a spatially variable increase in U in groundwaters of up to 2 μM. Concentrations of U in injection well sediment decreased from 10.3 g/kg to 4.64 g/kg; decreases were also observed in nearby monitoring wells, but concentrations of U in sediments actually increased in monitoring wells further away from the injection well. Ethanol additions were then resumed, restoring U(VI)-reduction and maintaining <0.1 μM U in groundwater. At the end of the trial, between 60 and 80% of U in monitoring well sediments was present as U(IV).

3.4.2. Reoxidation by exposure to nitrate

Proposed mechanisms of U(IV) reoxidation by nitrate include: abiotic oxidation by denitrification intermediates e.g. nitrite; direct oxidation by bacteria coupled to nitrate reduction; or oxidation by Fe(III) generated through oxidation by denitrification intermediates or by bacteria coupled to nitrate reduction (Senko et al., 2002). Nitrite alone was found to be a relatively poor oxidant of U(IV) compared to Fe(III) oxyhydroxides, but in combination with Fe(II) lead to complete reoxidation of U(IV), with the Fe(II) acting as an electron shuttle or catalyst between nitrate reduction and U(IV) oxidation (Senko et al., 2005). Amorphous biotic Fe(III) produced by biogenic nitrite oxidised U(IV) at a greater rate and extent, compared to the more crystalline biogenic Fe(III) with a lower surface area.

Nitrate-reducing bacteria appear to be particularly important in mediating the reoxidation of U(IV) by nitrate. A *Pseudomonas* species was isolated from a nitrate reoxidation system; total reoxidation of U(IV) occurred when *Pseudomonas* cells and nitrate were added to sterile pre-reduced sediment microcosms, but no reoxidation occurred when just nitrate was added to sterile systems (Wilkins et al., 2007). *Thiobacillus denitrificans* was observed to oxidise synthetic and biogenic uraninite under anaerobic conditions coupled to nitrate reduction (Beller, 2005). Reoxidation of U(IV) was investigated using two enrichment cultures from the Oak Ridge site; an iron(III)-reducing culture dominated by *Clostridium* spp. and a sulfate-reducing culture dominated by *Desulfovibrio* spp. (Boonchayaanant et al., 2009). In these systems, 5 mM nitrate failed to reoxidise U(IV) in both enrichment cultures. The concentration of nitrate in the sulfate-reducing system remained constant; attributed to the lack of nitrate-reducing bacteria. In contrast in the Fe(III)-reducing system, the 5 mM nitrate had decreased to almost zero after 48 h, although no nitrite was detected nor increases in ammonium concentration were observed. Lack of U(IV) reoxidation is proposed to be due to the absence of nitrate-reducing bacteria or the redox buffering effect of Fe(II) and/or sulfide. Indeed, the authors highlight work showing that *Clostridium* species can bioreduce U(VI) in the presence of nitrate (Madden et al., 2007).

There does not appear to be an obvious trend between the amount of U(IV) remobilised compared to the amount of nitrate added. For example, adding an 80-fold stoichiometric excess of nitrate to the influent of columns containing bioreduced Rifle sediments remobilised 97% of uranium over 54 days (Moon et al., 2007). A later column study by the same authors found the addition of nitrate reoxidised more U(IV) than dissolved oxygen, due to the faster reaction kinetics of oxygen with iron sulfides causing slower advancement of the reaction front through the column compared to nitrate, therefore protecting more U(IV) from contact with the oxidant (Moon et al., 2009). Perhaps comparable are results demonstrating a high proportion of uranium (around 86%) being reoxidised 10 days after exposure to a 1000-fold stoichiometric excess of nitrate in microcosm experiments (Wilkins et al., 2007). In contrast, another microcosm study found minimal (3%) reoxidation of U(IV) had occurred 20 days after exposure to a 240-fold stoichiometric excess of nitrate (Law et al., 2011). The mechanism for U retention in this system was unidentified as more than 80% of Fe(II) was oxidised to Fe(III) and nitrate reduction was observed.

Nitrate (2 mM) was added in situ to sediments containing bioreduced U(IV) at the Oak Ridge field site (Wu et al., 2010). Initially Fe(II) and sulfate were released to solution, then Fe(II) concentrations decreased presumably as Fe(III) oxyhydroxides precipitated. Up to 1 μM U was remobilised, concurrent with nitrite formation (full denitrification processes were observed). Subsequent additions of ethanol caused a transient increase in U(VI) up to around 2.5 μM, probably due to desorption as Fe(III) oxyhydroxides were reduced, before concentrations decreased to less than 0.1 μM.

In summary, the potential for U(IV) reoxidation must be carefully considered when deciding whether bioreduction offers a long-term remediation strategy for removing U(VI) from groundwater. Maintaining reducing conditions and/or continual electron donor supply may be required for long-term success. The presence of iron sulfides appears to play an important role in protecting U(IV) from reoxidation. The method of assessing the susceptibility of U(IV) to reoxidation is crucial, with lower amounts of remobilisation observed during more realistic experiments (e.g. microcosms > columns > field trials) perhaps due to preferential flow pathways developing in larger scale trials. It is important to consider the likelihood of reoxidation scenarios actually occurring within the decision making process.

4. Uranium phosphate biomineralisation

Although less extensively studied compared to bioreduction, uranium sequestration as insoluble uranyl U(VI) phosphate biominerals is another promising technique for in situ bioremediation, particularly for sites where bioreduction might be unfeasible due to high nitrate concentrations or where these is a risk of reoxidation occurring.

4.1. Early work & mechanisms

The phosphatase activity of *Serratia* sp. strain N14 has been exploited to remove U(VI) from solution (Macaskie et al., 1992). When supplied with organic phosphate donors such as glycerol

phosphates, this *Serratia* sp. over-produced phosphatases which liberated orthophosphate (inorganic phosphate, P_i) and precipitated with U(VI) as uranyl phosphate at the cell surface. In a flow-through bioreactor with cells immobilised in polyacrylamide gel, 9 g of uranium was precipitated per gramme of bacterial dry weight after a three week period; indeed the experiment was so successful it had to be stopped because of blockage by accumulated metal (Macaskie, 1990). The precipitate was identified as hydrogen uranyl phosphate [HUO_2PO_4] (HUP). Analysis of peptide fragments identified the enzyme responsible as a class PhoN phosphatase, which is a non-specific acid phosphatase (NSAP) (Macaskie et al., 1994a). Detailed investigations noted phosphatase enzymes from the *Serratia* strain were mostly localised in the periplasm, with some associated with the outer membrane and some found extracellularly (Jeong et al., 1997). Higher concentrations of phosphatase were present in the polar regions of the cell, as was accumulated uranyl phosphate. The authors suggested that the architecture of the cell surface prevents fouling by the biominerals, allowing uranyl to access to the inner and outer cell membrane. Later work suggested that lipopolysaccharides produced by phosphatases provide the initial nucleation site for metal deposition, with further uranyl phosphates juxtaposed to create 'tethered' metal phosphates, so preventing fouling of the cell surface (Macaskie et al., 2000). The phosphatase activity of *Serratia* has been shown to be tolerant to gamma radiation at doses up to 1368 Gy, suggesting a potential for use at nuclear sites (Paterson-Beedle et al., 2012).

Phosphatase activity is a common feature of almost all microorganisms. P_i is the preferred source of the essential nutrient phosphorus for bacteria. If a surplus is present some organisms bioaccumulate P_i and store it intracellularly as polyphosphate granules; if it is in short-supply then a specific transport system can be used to ensure sufficient uptake (Hirota et al., 2010). Under anoxic conditions, some organisms such as *Acinetobacter* can then use the polyphosphate granules as an energy source via hydrolysis and efflux of phosphate (van Groenestijn et al., 1988). The impact of this process on metal cycling is currently poorly defined. In the absence of P_i, alternative sources of P can be transformed by bacteria to release P_i, including organophosphates (via hydrolytic cleavage catalysed by phosphatases), inorganic phosphite (via enzymatic oxidation) and phosphonates (via cleavage catalysed by C–P lyases). This action is an essential part of the phosphorus cycle. Some bacteria can transform organically bound phosphorus — that otherwise would not be used by other organisms — into an accessible form, for example from nucleic acids (Siuda and Chrost, 2001), phytate (Lim et al., 2007) or phospholipids (Ko and Hora, 1970). Up to 80% of the soil microbial population are able to accomplish hydrolytic cleavage of organophosphates through phosphatase activity, including *Bacillus*, *Serratia*, *Proteus*, *Arthrobacter* and *Streptomyces* species and various fungi (Ehrlich, 1990).

However, not all bacteria capable of phosphatase activity are able to remove uranium from solution; enterobacteria with acid-phosphatases were unable to do so (Macaskie et al., 1994a). This was suggested to indicate a *Serratia* strain-specific cell architecture may be necessary. More recently, uranium biomineralisation via phosphatase activity has been demonstrated using environmental isolates of *Rahnella*, *Bacillus*, and *Aeromonas* species; and the indigenous soil bacterial community from the US DOE Oak Ridge site (Beazley et al., 2007; Martinez et al., 2007; Shelobolina et al., 2009). Genetically altered strains of bacteria are also able to precipitate uranyl phosphates (Martinez et al., 2007), including engineered strains of *Deinococcus radiodurans* (Appukuttan et al., 2007), *Escherichia coli* with added acid-phosphatase genes (Basnakova et al., 1998), and strains of *Pseudomonas veronii* and *Pseudomonas rhodesiae* with added alkaline-phosphatase genes (Powers et al., 2002).

4.2. Mineralogical endpoints

The end-products are reported to be U(VI) phosphate minerals; these are insoluble and do not undergo redox changes. Experiments with *Serratia* sp. strain N14 produced HUP (Macaskie et al., 1992). Adding ammonium acetate to the growth solution led to $NH_4UO_2PO_4$ being formed, which has a lower solubility product than HUP (Yong and Macaskie, 1995). Experiments using soils from the Oak Ridge site found autunite minerals were precipitated (Beazley et al., 2007), while when an environmental isolate was used the uranium was incorporated into hydroxyapatite [$Ca_5(PO_4)_3OH$] which is much less soluble at near-neutral pH compared to autunite (Shelobolina et al., 2009). Hydroxyapatite is argued to be a preferable end product because a large area of hydroxyapatite with dilute U(VI) concentrations should be more stable to dissolution over longer periods of times than a smaller area of autunite with higher concentrations of U(VI).

4.3. Demonstration at the US DOE Oak Ridge site, Tennessee

Uranium biomineralisation via phosphatase activity has been investigated for potential use at the Oak Ridge site, particularly due to the presence of acidic soils with high nitrate concentrations that may inhibit bioreduction (Beazley et al., 2007; Martinez et al., 2007). Screening of 135 environmental isolates identified 85 to be phosphatase-positive. Initial experiments with uranyl acetate and glycerol-3-phosphate (G3P) determined that a five-fold molar excess of phosphate would be sufficient to precipitate uranyl from solution. The ability of three representative strains to biomineralise 200 μM U was investigated, excluding carbonate to simulate site groundwater conditions. The *Bacillus* and *Rahnella* strains were able to liberate P_i in the presence of G3P which precipitated 73% (*Bacillus*) and 95% (*Rahnella*) of uranyl from solution. The phosphatase-negative *Arthrobacter* strain was unable to remove uranyl from solution; although the cells did not grow they remained culturable. The optimum pH was reported to be 5.0–5.5, suggesting non-specific acid phosphatases were responsible; this was confirmed by molecular genetic analysis. The culturability of the *Rahnella* cells decreased significantly on exposure to uranium, but recovered by the end of the experiment (after 3.5 days), while the *Bacillus* and *Arthrobacter* species were not affected. The U-phosphate precipitate was identified as calcium autunite [$Ca(UO_2)_2(PO_4)_2$].

Subsequent work determined that the *Rahnella* strain is able to biomineralise uranyl to chernikovite [$H_2(UO_2)_2(PO_4)_2$] under anaerobic conditions and in the presence of high nitrate (Beazley et al., 2009). The cells were able to respire nitrate in the absence of oxygen, although they grew more slowly and released less P_i compared to in aerobic conditions. A stress response was observed in both the aerobic and anaerobic experiments, but despite this 95% of uranium was removed from solution after 120 h incubation. Post uranium removal the cells in aerobic conditions recovered but those in anaerobic conditions did not. This was suggested to be due to the toxic effects of nitrite produced from nitrate respiration (the *Rahnella* sp. was unable to denitrify) in combination with the toxic effects of uranium. TEM images showed that uranium was initially associated with the cell surfaces, but over time it desorbed and precipitated extracellularly. This could be due to an initial reaction with the Gram-negative outer membrane/lipopolysaccharides before sufficient P_i generation, or due to the cells acting as a nucleation surface for biomineral precipitation.

Another experiment used pure cultures of bacteria isolated from sediments in an area with high levels of dissolved calcium (Shelobolina et al., 2009). Three strains, 99% similar to *Aeromonas hydrophila*, *Pantoea agglomerans* and *P. rhodesiae* were isolated which could remove uranium from solution under aerobic and nitrate-reducing conditions coupled to G3P hydrolysis. Analysis of the precipitate generated by the isolate closely related to *A. hydrophila* showed that uranium was incorporated within the mineral structure of hydroxyapatite. Approximately 16% of uranium was solubilised during five washing cycles meaning the majority was sequestered in the mineral phase. The authors explained that the combination of the high concentrations of Ca and circumneutral pH lead to hydroxyapatite precipitation rather than the Ca-autunite formed in previous experiments (Beazley et al., 2007).

Experiments with flow-through columns containing site sediment and synthetic groundwater amended with G3P demonstrated 97% removal of 200 μM U(VI) at pH 5.5 and 7.0 (Beazley et al., 2011). Most of the uranium was precipitated within 1 cm of the inlet. Control columns without glycerol phosphate removed 88–95% of the U(VI), presumably through sorption. Sequential extractions provided evidence for uranyl phosphate precipitation. XAS identified the uranium speciation as U(VI), which was mainly precipitated as uranyl phosphate minerals at pH 5.5, while at pH 7 it was mostly adsorbed to iron oxides with minor occurrences of uranyl phosphate precipitation. Similar results were obtained in anaerobic microcosm experiments (Salome et al., 2013). Almost all of the uranium and P_i released from G2P metabolism sorbed to sediments; XAS identified some U(VI)-phosphate but no evidence for U(VI) reduction was observed.

4.4. Limitations

All of the studies which demonstrate uranium biomineralisation via phosphatase activity have used glycerol phosphate as a carbon and phosphate source, either as glycerol-3-phosphate or glycerol-2-phosphate depending on commercial availability. Glycerol phosphate may not be cost effective for uranium biomineralisation (Roig et al., 1995; Lloyd and Macaskie, 2000). Alternative phosphate donors were tested for the *Serratia* system, but the enzyme in this organism was found to be substrate-specific (Michel et al., 1986). Bioreactors containing *E. coli* were able to liberate phosphate from phytic acid, which precipitated with uranyl nitrate as HUP, highlighting the potential for use of plant wastes as a source of P_i (Paterson-Beedle et al., 2010). Tributylphosphate (TBP), a solvent to extract actinides during nuclear fuel reprocessing, has been investigated as an alternative source of carbon and phosphorus in enrichment cultures (Thomas and Macaskie, 1996). A mixed culture containing *Pseudomonas* spp. was able to degrade TBP in the presence of U(VI), liberating 1-butanol for growth and releasing P_i which precipitated as uranyl phosphates. However, it is questionable whether introducing a solvent to groundwater would be considered a responsible remediation strategy.

Some evidence exists which indicates that if a system is phosphate-limited, bacteria can cause dissolution of uranyl phosphates such as autunite (Smeaton et al., 2008; Katsenovich et al., 2012). The ability of bacteria to reduce U(VI) in uranyl phosphate minerals has recently been assessed (Rui et al., 2013). Biogenic HUP associated with *Bacillus subtilis* and freely suspended abiotic HUP were incubated with dissimilatory metal-reducing bacteria in bicarbonate or HEPES buffer, with or without phosphate. U(IV) was produced, either in the form of adsorbed monomeric U(IV) or as an amorphous solid similar to ningyoite. The authors considered whether this was formed from solid phase U(VI) reduction, in which case the reduction rate should be proportional to HUP surface area, or dissolved phase U(VI) reduction where reduction rate would be proportional to the dissolution rate. A greater extent of reduction was observed with biogenic HUP, which had an effective surface area 27 times greater than abiotic HUP. More U(VI) reduction was observed in the presence of bicarbonate (which promotes HUP dissolution), while a lower extent of reduction was observed with phosphate. This suggests bacterial reduction of dissolved U(VI) rather than solid phase U(VI) in HUP, with precipitation of U(IV) driving further dissolution by disturbing the equilibrium between HUP and $U(VI)_{(aq)}$. Similar dissolution controlled reduction of solid U(VI) has been observed using synthetic sodium boltwoodite (Liu et al., 2006) and intragrain sodium boltwoodite in contaminated Hanford sediments (Liu et al., 2009).

5. Other priority radionuclides

5.1. Technetium

Technetium ^{99}Tc is a long lived (half-life 212,000 years), high yield radioactive fission product produced as part of the nuclear fuel cycle. Consequently it has contaminated groundwater at nuclear sites such as the US DOE Hanford site and Sellafield in the UK. Under oxic conditions in the natural environment, it is mobile as the highly soluble pertechnetate ion ($Tc(VII)O_4^-$) and is of concern both as a mobile radioactive contaminant and as a bioavailable analogue for sulfate (McBeth et al., 2007; Icenhower et al., 2010). Under reducing conditions it can form insoluble and strongly sorbing hydrous $Tc(IV)O_2$ phases. Therefore, bacterially-mediated reduction offers a promising strategy for removing soluble Tc(VII) from contaminated groundwater across a wide range of concentrations and has been demonstrated in laboratory experiments e.g. Law et al. (2010a) and Wilkins et al. (2007) and in the field (Istok et al., 2004).

5.1.1. Early work & mechanisms

Two mechanisms of Tc(VII) bioreduction have been identified; direct enzymatic reduction by microbial hydrogenases and indirect reduction by biogenic Fe(II) or sulfide (Lloyd et al., 2000a; Burke et al., 2005; McBeth et al., 2007). Given the low concentrations of Tc(VII) in the environment, indirect reduction by Fe(II) is likely to be the dominant mechanism (Lloyd et al., 1999a; McBeth et al., 2007), apart from in sediments with very low iron concentrations (Wildung et al., 2004). Indeed, novel gamma camera imaging techniques have shown Tc(VII) removal at picomolar concentrations (below the solubility limit for hydrous TcO_2-like phase formation), and a direct link between biogenic Fe(II) and Tc(IV) (Lear et al., 2010; Vandehey et al., 2012). Much greater quantities of Tc(VII) were removed by *Geobacter*, *Anaeromyxobacter* and *Shewanella* in the presence of ferrihydrite compared to experiments with just cells, highlighting the importance of biogenic Fe(II) in Tc(VII) bioreduction (Plymale et al., 2011).

A number of bacteria have been identified that enzymatically reduce Tc(VII) coupled to the oxidation of H_2 or certain organic compounds (Fredrickson et al., 2004) including: the metal reducers *Geobacter* spp. (Lloyd and Macaskie, 1996; Lloyd et al., 2000a), *S. putrefaciens* (Wildung et al., 2000) and *A. dehalogenans* strain 2CP-C (Marshall et al., 2009); sulfate-reducing *Desulfovibrio* spp. (Lloyd et al., 1999b; De Luca et al., 2001); haloalkaliphilic *Halomonas* (Khijniak et al., 2003); acidophilic *Thiobacillus* spp. (Lyalikova and Khizhnyak, 1996); and *E. coli* (Lloyd et al., 1997). Electron transfer is mediated by periplasmic hydrogenase enzymes (Lloyd et al., 1997, 1999a,b; De Luca et al., 2001) although cytochromes may also facilitate electron transfer in *S. oneidensis* MR1 (Marshall et al., 2008).

Alternatively, abiotic reduction of Tc(VII) to Tc(IV) is possible, facilitated by Fe(II) biominerals generated from reduction of Fe(III) oxides (Lloyd et al., 2000a; Fredrickson et al., 2004). Biogenically produced magnetite was able to completely remove Tc(VII) from solution, while bio-vivianite and bio-siderite removed 68 and 84% respectively (McBeth et al., 2011). Abiotic reduction via Fe(II) minerals have now been documented in: the clay minerals nontronite (Jaisi et al., 2009; Yang et al., 2012) montmorillonite, nontronite, rectorite, mixed layered illite–smectite, illite, chlorite, and palygorskite (Bishop et al., 2011); amorphous iron sulfide (Liu et al., 2008); mackinawite (Wharton et al., 2000), Fe(II) sorbed to aluminium hydroxides (Peretyazhko et al., 2008); and in naturally reducing zones of aquifers containing Fe(II) minerals including Fe(II)-phyllosilicates, pyrite, magnetite and siderite (Peretyazhko et al., 2012). This is likely to be a dominant pathway for reduction in many environmental scenarios. Abiotic reduction of Tc(VII) by aqueous Fe(II) was found to be strongly pH dependent; complete and rapid removal of Tc(VII) was observed at pH 7 and 8, but not at pH 6 (Zachara et al., 2007).

5.1.2. Reduction and reoxidation studies

Sediment microcosms spiked with pertechnetate were able to completely remove Tc(VII) from solution during Fe(III)-reduction and precipitate hydrous TcO_2 (Burke et al., 2005). Removal of pertechnetate has been demonstrated in microcosm experiments using sediment from nuclear sites (McBeth et al., 2007; Wilkins et al., 2007; Begg et al., 2008;

Morris et al., 2008). The presence of nitrate and manganese is generally considered to inhibit the development of metal-reducing conditions. Tc(VII) removal was only observed after Fe(III)-reducing conditions had developed, and the presence of 100 mM nitrate prevented this from occurring (McBeth et al., 2007). Although acidic sediments failed to develop Fe(III)- and Tc(VII)-reducing conditions, denitrification of 10 mM nitrate caused the pH to rise from 5.5 to 7.2 which allowed progression to Fe(III)-reduction and Tc(VII) removal (Law et al., 2010a; Geissler et al., 2011). This change in pH did not occur with lower concentrations of nitrate. Tc removal has been reported under nitrate-reducing conditions in selected experiments (Fredrickson et al., 2004; Istok et al., 2004; Eagling et al., 2012).

Exposure of bioreduced sediments to air caused reoxidation of 40 to 80% Tc; the remaining amount was identified as a recalcitrant hydrous $Tc(IV)O_2$ solid phase and/or incorporated into new mineral phases formed on reoxidation (McBeth et al., 2007; Begg et al., 2008; Morris et al., 2008). The rate of Tc(VII) reduction and the susceptibility of bioreduced Tc(IV) to reoxidation via oxygen exposure have been investigated in sediments of different lithology (Fredrickson et al., 2009). Tc(VII) reduction was considerably faster in a fluvial sediment from Hanford compared to a saprolite from Oak Ridge, despite the saprolite containing a higher concentration of Fe(II). This may be because the saprolite contained Fe(II) associated with sheet silicate minerals, which is slower to react with pertechnetate compared to the Fe(II) sorbed to Fe(III) oxides in the fluvial sediment. Alternatively it could be due to mass transfer limitations in clayey sediments. Tc(IV) in the fluvial sediment was oxidised rapidly and completely on exposure to oxygen, but reoxidation was slow and incomplete in the clayey saprolite. These differences may be due to Tc(IV) forming reoxidation-resistant aggregates associated with Fe-containing mica (perhaps celadonite) in the saprolite. Biogenic TcO_2 has been shown to be susceptible to reoxidation by Mn(III/IV) oxides in anoxic but unreduced sediments (Fredrickson et al., 2004, 2009), although how this situation would arise in the natural environment is unclear. While humic acids increased the rate and extent of oxidative dissolution of TcO_2, the presence of EDTA decreased both (Gu et al., 2011). In contrast to oxygen exposure, addition of nitrate failed to remobilise Tc(IV) (McBeth et al., 2007; Wilkins et al., 2007; Begg et al., 2008; Morris et al., 2008). Failure of some Tc(IV) to be reoxidised may be due to it being protected by newly precipitated Fe(III) oxides (Zachara et al., 2007).

In summary, reduction of Tc(VII) facilitated by biogenic Fe(II) appears to be a promising bioremediation strategy for removing Tc from groundwater and fixing into insoluble minerals that are mostly resistant to oxidative remobilisation. Important factors to consider are: sediment mineralogy, with clayey sediments less susceptible to reoxidation; and sediment pH, as slightly acidic conditions appear to inhibit Tc removal.

5.2. Neptunium

Neptunium is an actinide produced by the decay of plutonium and americium. Although it is not a widespread groundwater contaminant at nuclear sites, it is of concern in nuclear wastes due to its high radiotoxicity and the long half-life (2.13×10^6 years) of its dominant isotope ^{237}Np. Neptunium exists as the neptunyl cation $Np(V)O_2^+$ in a wide range of environmental conditions; it sorbs relatively poorly to surfaces or microbial biomass and is therefore very mobile (Kaszuba and Runde, 1999). Np(IV) exists under reducing conditions, it is poorly soluble, has a strong tendency to form aqueous complexes and can be removed from solution by hydrolysis and reaction with surfaces.

Np(V) can be removed from solution via a combined bioreduction–biomineralisation system using anaerobic bacteria (Lloyd et al., 2000b). *S. putrefaciens* was able to reduce Np(V) to Np(IV), which then precipitated with phosphate liberated by phosphatase activity of a *Serratia* sp. Np(V) did not precipitate with *Serratia*-generated phosphate, or when solely reduced to Np(IV), hence a coupled system was required. Another study found that an anaerobic microbial consortium supplied with hydrogen or pyruvate as an electron donor was able to reduce Np(V) and precipitate it as Np(IV) (Rittmann et al., 2002). *G. metallireducens* and *S. oneidensis* were both able to reduce aqueous Np(V)-citrate to aqueous Np(IV)-citrate, while only *S. oneidensis* was able to reduce unchelated Np(V) to insoluble Np(IV) (Icopini et al., 2007). This confirms earlier observations, that *G. sulfurreducens* was unable to reduce $Np(V)O_2^+$ (Renshaw et al., 2005). As it reduced $U(VI)O_2^{2+}$ by a single electron transfer to U(V), which rapidly disproportionated to U(IV), this suggests it is unable to transfer electrons to pentavalent actinides. This demonstrates the potentially important role played by organic ligands; they can make Np(V) less toxic to bacteria but allow Np(IV) complexes to remain in solution. Similar observations have been made for Cr(VI) in bioreducing systems (Mabbett et al., 2002). Microbially-active sediment systems were able to facilitate Np(V) reduction to Np(IV) when supplied with acetate as an electron donor (Law et al., 2010b). In a similar mechanism to that demonstrated for technetium, bioreduced Fe(II) in sterile sediments was shown to abiotically reduce Np(V) to poorly soluble Np(IV), suggesting abiotic reduction is possible for Np(V). Reoxidation experiments demonstrated the sediment-associated Np(IV) was somewhat resistant to remobilisation.

5.3. Plutonium

Plutonium is a long-lived toxic actinide produced by neutron activation of uranium. Kilogramme quantities of ^{239}Pu (half-life 24,000 years) and ^{240}Pu (half-life 6560 years) have been released into the environment (Morris et al., 2001). Plutonium has complex redox chemistry. The dominant oxidation state in most environments is Pu(IV), which forms a highly insoluble hydrous oxide $Pu(OH)_4$ and sorbs strongly to colloidal and suspended material (Banaszak et al., 1999; Choppin et al., 2002). In oxidising conditions, the most significant soluble state is the plutonyl cation $Pu(V)O_2^+$, which has a lower tendency to be sorbed compared to Pu(IV) (Choppin, 2007). Under conditions relevant to natural waters, Pu can exist in multiple oxidation states simultaneously, meaning small changes in pH and redox can lead to changes in speciation and environmental mobility (Ewing, 2010).

Results from a study of porewaters indicated seasonal cycles in Fe, Mn and Pu may be influenced by non-redox driven microbial processes (Morris et al., 2001). Three laboratory studies have investigated the role played by Fe(III)-reducing bacteria *G. metallireducens* and *S. oneidensis* in Pu reduction (Boukhalfa et al., 2007; Icopini et al., 2009; Renshaw et al., 2009). Both bacteria could reduce aqueous Pu(V)/(VI) to insoluble nanocrystalline Pu(IV), while soluble Pu(III) was not produced. When supplied with amorphous $Pu(IV)OH_4$, *S. oneidensis* produced minor amounts of Pu(III) but *G. metallireducens* produced little (Boukhalfa et al., 2007). In contrast, both bacteria were able to rapidly reduce soluble Pu(IV)-EDTA to Pu(III)-EDTA, highlighting the importance of complexing ligands on Pu biogeochemistry. Other bacteria shown to reduce Pu(IV) to Pu(III) include *Clostridium* sp. (Francis et al., 2008), *Bacillus* sp. (Rusin et al., 1994), and *Bacillus mycoides* and *Serratia marcescens* (Luksiene et al., 2012). *Shewanella alga* reduced $Pu(V)O_2^+$ to amorphous $Pu(III)PO_4$ (Reed et al., 2007; Deo et al., 2011). Finally, plutonium in contaminated sediments remained remarkably resistant to solubilisation throughout a cascade of anaerobic processes, including fermentation and Fe(III) reduction (Kimber et al., 2012).

5.4. Americium

Americium is an actinide produced by neutron activation of plutonium; ^{241}Am is the most common isotope with a half-life of 433 years. It exists as Am(III) under environmentally relevant conditions including in natural waters and is not subject to redox transformation (Choppin et al., 2002; Siegal and Bryan, 2003; Ewing, 2010). Americium readily sorbs to soils and sediments, consequently it has limited mobility in the environment and is not a target for in situ bioremediation. For completeness, documented biogeochemical interactions include biosorption

to marine algae (Fisher et al., 1983) and biomineralisation with biogenic phosphate produced by a *Serratia* sp. (Macaskie et al., 1994b).

5.5. Iodine

Radioactive iodine is produced as a fission product, with the isotope ^{129}I being of particular concern due to its long half-life (15.7×10^6 years), mobility in the environment and bioavailability. Iodine has a complex biogeochemistry with iodide (I^-), molecular iodine (I_2) and iodate (IO_3^-) all stable under environmental conditions, mobile in natural waters and forming a range of organic complexes (Gallard et al., 2009; Fox et al., 2010; Kaplan et al., 2011; Shetaya et al., 2012). Mobile iodide predominates under reducing conditions, whereas in oxidising conditions iodate can be present and interact with organic matter and clays (Hu et al., 2007).

A wide range of soil organisms can convert iodide to volatile methyl iodide (CH_3I), and in some cases this activity is enhanced by the addition of organic carbon such as glucose (Amachi et al., 2003). Volatilisation of radioiodine from soils was confirmed using ^{125}I, while the role of microbes was confirmed as volatilisation was inhibited by the addition of bacterial-specific antibiotics. *D. desulfuricans* was able to enzymatically reduce iodate to iodide in bicarbonate and HEPES buffers, while *S. putrefaciens* was only able to perform this transformation in HEPES (Councell et al., 1997). As Fe(II), sulfide and FeS were shown to abiotically reduce iodate to iodide, it is inferred that Fe(III)- and sulfate-reducing bacteria are able to mediate iodate reduction both directly and indirectly.

Iodine speciation and transport was studied using representative surface soils and sediments collected at US nuclear facilities (Hu et al., 2007). Approximately 90% of iodine was present as organic species in soils, while inorganic iodine was important (up to 50%) only in sediments with low organic matter. An earlier study demonstrated the complex biogeochemical behaviour of iodine and emphasised the importance of structural Fe(II) in some clay minerals in mediating iodate reduction to iodide (Hu et al., 2005). The influence of iron minerals on iodine was further illustrated by data showing in situ bacteriogenic Fe(III) oxides at Chalk River, Canada were able to sorb 54% of iodine and 75% of ^{129}I from waters at near-neutral pH (Kennedy et al., 2011). Field tests demonstrated that iodide injected into the oxic zone of an aquifer was oxidised to iodine and iodate (Fox et al., 2010). Transport of iodate injected into an oxic aquifer zone was retarded, while iodate injected into an Fe(III)-reducing zone was rapidly reduced to iodide.

5.6. Strontium and caesium

Radioactive strontium and caesium are produced as fission products. Both have stable isotopes which are naturally occurring and ubiquitous in the natural environment. Neither strontium nor caesium are redox sensitive so their fate in the environment is mostly influenced sorption, although biotic interactions may play a role, for example by changing the pH or producing soluble ligands or new biomineral phases.

Several studies have demonstrated strontium biomineralisation via precipitation of strontium carbonate or calcite by actively metabolising microorganisms including *Pseudomonas fluorescens* (Anderson and Appanna, 1994), epilithic cyanobacteria (Ferris et al., 1995), *Halomonas* (Achal et al., 2012), *Sporosarcina pasteurii* (formerly *Bacillus pasteurii*) (Ferris et al., 2004; Fujita et al., 2004; Cuthbert et al., 2012), and indigenous groundwater bacteria (Fujita et al., 2010; Tobler et al., 2011). In most cases this occurs through carbonate generation linked to the hydrolysis of urea by ureolytic bacteria in the presence of calcium. Alternative bioremediation techniques proposed for strontium include sequestration as $(Ba,Sr)SO_4$ by desmid green algae (Krejci et al., 2011), incorporation into biogenic hydroxyapatite produced by a *Serratia* sp. (Handley-Sidhu et al., 2011a,b) and co-treatment with Tc(VII) and high nitrate levels via microbially induced increases in pH and alkalinity during bioreduction of Fe(III) and nitrate (Thorpe et al., 2012b).

Pure culture studies have shown that microbial biosorbents are inefficient for caesium uptake, but uptake by actively metabolising microorganisms is more efficient (Macaskie, 1991). Both K^+ and Cs^+ are taken up by the same metabolism-dependent transport systems due to the similarity of the cations. However in sediment systems, the mobility of Cs in the environment is dominated by cation-exchange processes at mineral surfaces, especially with phyllosilicates where Cs can form inner sphere complexes at edge sites. Indirect microbial impacts such as the release of competing ammonium ions or changes in mineral stability may play a role in controlling Cs mobility (Brookshaw et al., 2012).

6. Conclusions and future directions

Much research has been done on uranium biogeochemistry and bioremediation. Employment of bioreduction in particular appears promising, with state-of-the-art molecular techniques being developed to monitor progress and refine its application in the field. Indeed, field trials have shown sustained removal of U(VI) from groundwater. Questions do still remain however about the longevity of bioreduced U(IV). Although biomineralisation has been demonstrated to generate poorly soluble uranyl phosphates using pure bacterial cultures, results with soils from the Oak Ridge site have been dominated by sorption effects. Of the other priority radionuclides, technetium may be amenable to treatment by bioreduction and strontium amenable to biomineralisation.

Areas where research should continue include assessing which mechanisms of bacterial electron transport dominate, in both the natural environment and during biostimulation trials. There is still work to be done to determine the precise mechanism(s) of electron transfer to U(VI) in circumneutral aquifer sediments. The reduction of U(VI) at alkaline pH, especially the role played by Gram-positive bacteria should be explored in more detail, as should establishing conclusively whether bacteria are able to enzymatically reduce solid phase U(VI). Another area for future research is determining the longevity of bioreduced U(IV). This could be assessed by doing reoxidation experiments as part of field biostimulation trials, but crucial understanding is lacking in the factors which cause monomeric U(IV) or uraninite to precipitate. It is clear that precipitation of monomeric U(IV) is favoured under the conditions present at the Rifle site, however, as monomeric U(IV) is absent from the geological record and laboratory experiments have not demonstrated unequivocal evidence for an ageing mechanism, it is uncertain whether monomeric U(IV) is specific to that particular site or of wider significance. Field studies at alternative sites and/or in other countries could address this. In particular, uranium contamination in groundwater at mining or milling sites is an area which is yet to be examined. Application of uranium-phosphate biomineralisation in the field could be trialled, while if widespread use is indeed limited by the cost of glycerol phosphate, alternative organic phosphorus substrates should be investigated.

Overall it is clear that microbial cycling processes have a significant impact on radionuclide behaviour across a wide range of environments and will be important in managing contaminated land sites as well as in geological disposal scenarios where biogeochemical processes are likely to occur and should be considered in safety case development.

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